DIETARY ESSENTIAL AMINO ACID REQUIREMENTS OF INDIAN MAJOR CARP, *CATLA CATLA* (HAMILTON) FINGERLING

ABSTRACT
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SUBMITTED FOR THE AWARD OF THE DEGREE OF

Doctor of Philosophy

IN

ZOOLOGY

BY

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ALIGARH (INDIA)

2013

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ABSTRACT

Indian major carp, Catla, *Catla catla*, the fish under study, is the fastest growing species among the three Indian major carps, the other two being rohu, *L. rohita*, and mrigal, *C. mrigala*. *C. catla*, a predominant zooplankton feeder, is an important component of polyculture with the above two species of carps. It has a great market demand because of its high nutritional value and good taste. Unavailability of nutritionally balanced commercial feeds for the concerned species is the most important factor hampering efforts in its culture. Hence, it is essential to develop nutritionally balanced quality feeds for the future development of aquaculture of this species. To ensure maximal growth, fish species need ten indispensible amino acids in their diet. There is little information available on essential amino acid requirements of *C. catla* which is the major impediment in producing nutritionally adequate manufactured feeds for its intensive culture. Also, the lack of consistent data on the essential amino acid requirements of different growth stages of this fish makes it difficult to correctly formulate diets that maximize growth to their full genetic potential. Hence, this study was undertaken to determine all the ten essential amino acid requirements of fingerling *C. catla*. The information generated during the course of the present study has been compiled in the form of this thesis.

For studying the arginine, lysine, isoleucine, valine, leucine, threonine, tryptophan, histidine, total sulphur amino acid and total aromatic amino acids requirements of fingerling *C. catla* Halver's amino acid test diet (isonitrogenous 33% crude protein and isocaloric 16.72 kJ/g gross energy) containing casein, gelatin and crystalline L-amino acids mixture with graded levels of test amino acid was used. For determining the requirement of each amino acid, triplicate groups of fish were randomly stocked at a density of twenty five fingerlings in 70 L indoor polyvinyl circular troughs fitted with a water flow-through system (1-1.5 L/min). The experimental diets were fed to fish to apparent satiation at 08:00, 12:30 and 17:30 h for 12 weeks.

Arginine has important nutritional and physiological roles and is established as indispensable in the diet of many fish species (NRC 2011). In addition to as an important component of protein, arginine is involved in several biologically important metabolic

THESIS

pathways. Dietary arginine requirement of fingerling *Catla catla* was determined by feeding casein-gelatin based isonitrogenous (33% crude protein) and isocaloric (16.72 kJ/g gross energy) amino acid test diets containing six graded levels of L-arginine (1%, 1.25%, 1.5%, 1.75%, 2% and 2.25% dry diet) for 12 weeks. Maximum absolute weight gain (AWG; 6.93 g/fish), protein efficiency ratio (PER; 2.13), protein deposition (PD; 0.36), arginine retention efficiency (ARE; 78%) and best feed conversion ratio (FCR; 1.42) were recorded in fish fed 1.75% arginine of the dry diet. Maximum carcass protein (15.57%) and RNA/DNA ratio (4.79) were also recorded for the group fed 1.75% arginine of the dry diet. Quadratic regression analysis at 95% maximum or minimum response of above growth parameters against varying levels of dietary arginine yielded the arginine requirement of fingerling *C. catla* at 1.67% of the dry diet. On the basis of above analysis of the growth parameters, it is recommended that inclusion of dietary arginine at 1.67% of the dry diet, corresponding to 5.06% dietary protein is optimum for formulating arginine-balanced, cost-effective quality feeds for the mass culture of fingerling *C. catla*.

Lysine is an essential amino acid present in high proportion in fish muscle tissue, involved in growth and maintenance of positive nitrogen balance, also used in "cross-linking" protein, especially collagen. A twelve-week experiment was conducted to quantify dietary lysine requirement of fingerling *Catla catla* by feeding casein-gelatin based diets (33% crude protein; 16.72 kJ/g gross energy) with six levels of L-lysine (1.25, 1.50, 1.75, 2.00, 2.25 and 2.50% dry diet). Live weight gain (LWG), feed conversion ratio (FCR), protein deposition (PD), lysine retention efficiency (LRE%) and RNA/DNA ratio were used as the response criteria. Second-degree polynomial regression analysis at 95% maximum and minimum response of LWG and FCR data against varying levels of dietary lysine exhibited the lysine requirement between 1.8-1.9% dry diet, corresponding to 5.5-5.7% dietary protein. Regression analysis of PD, LRE and RNA/DNA ratio yielded the requirement between 1.7-1.8% dry diet, corresponding to 5.2-5.5% dietary protein. Since live weight gain and protein deposition are the key parameters for estimating nutrient requirement, these tools were used to recommend the lysine requirement of fingerling *C. catla* which ranges between 1.7-1.8% dry diet.

Evaluation of isoleucine requirement is of particular importance because isoleucine along with the other two branched-chain amino acids acts as nutrient regulator of protein synthesis and protein degradation. It is also involved in the insulin biosynthesis and secretion. In addition to this, it helps in energy production in the body and has been found to reduce twitching and tremors in animals. In order to determine the dietary isoleucine requirement of fingerling catla, *Catla catla*, six isonitrogenous (33% crude protein) and isocaloric (16.72 kJ/g gross energy) amino acid test diets containing casein, gelatin and crystalline L-amino acids with graded levels of isoleucine (0.5, 0.75, 1.0, 1.25, 1.5 and 1.75% of the dry diet) were prepared. Growth of the fish was found to increase with the incremental levels of isoleucine up to 1.25% of the dry diet. Quadratic regression analysis at 95% maximum response of absolute weight gain (6.18 g/fish), protein productive value (0.32), isoleucine retention efficiency (71.91%), RNA/DNA ratio (4.81) and carcass protein (15.7%) against varying levels of dietary isoleucine yielded the requirement in the range of 1.13-1.18% of the dry diet, corresponding to 3.42-3.58% dietary protein.

Valine is one of the indispensable amino acid which is involved in many metabolic activities. It is required for the repair and growth of tissues, and also for the maintenance of nitrogen balance in body. A 12-week feeding-trial was conducted to determine the dietary valine requirement of fingerling *Catla catla*. Seven casein-gelatin based diets (33% crude protein; 16.72 kJ/g gross energy) containing graded levels of valine (0.51, 0.69, 0.91, 1.12, 1.31, 1.49 and 1.71% dry diet) were fed to triplicate groups of fish to apparent satiation. Absolute weight gain (AWG), protein productive value (PPV), valine gain (VG), feed conversion ratio (FCR) and carcass protein improved significantly ($P<0.05$) with the increasing concentrations of dietary valine from 0.51 to 1.12%. Quadratic regression analysis of AWG, PPV, VG and carcass protein at 95% maximum ($Y_{95\%max}$) response against varying levels of dietary valine yielded the requirement at 1.04, 1.03, 1.04 and 0.98% of dry diet, respectively. Based on above analysis, it is recommended that inclusion of valine at 1.02% of dry diet, corresponding to 3.09% dietary protein is optimum in formulating valine-balanced feeds for fingerling *C. catla*.

Leucine, a member of aliphatic side chain amino acid family is essential for normal growth and reproductive potential of the fish. It plays an important role in protein synthesis, promotes insulin release, and inhibits protein degradation. This study was aimed at quantifying leucine requirement of fingerling *Catla catla* by conducting a 12-week feeding trial. Six casein-gelatin based (33% crude protein, 16.72 kJ/g gross energy) amino acid test diets containing different concentrations of leucine (0.73, 0.97, 1.24, 1.46, 1.74 and 1.97% dry diet) were fed to triplicate groups of fish. Maximum absolute weight gain (AWG, 7.45 g/fish), protein gain (PG, 1.31 g/fish), leucine gain (LG, 85.33 mg/fish), RNA/DNA ratio (4.62) and best feed conversion ratio (FCR, 1.51) were recorded at 1.74% dietary leucine. Hematological characteristics were also found to be optimum in fish fed diet with 1.74% leucine. Quadratic regression analysis at 95% maximum response of AWG, PG, LG, RNA/DNA ratio and minimum response of FCR against dietary leucine concentrations reflected the requirement at 1.58, 1.59, 1.58, 1.57 and 1.57% dry diet, respectively. Based on above results, inclusion of leucine ranging from 1.57-1.59% of the dry diet, corresponding to 4.76-4.82% dietary protein is recommended for developing leucine-balanced commercial feeds for the intensive culture of *C. catla.*

Threonine participates in protein synthesis, and its catabolism generates many products important in metabolism. It acts as a precursor of glycine and serine, is involved in immune responses, needed in gastrointestinal mucin production. A 12-week feeding trial was conducted to determine the dietary threonine requirement of fingerling Indian major carp, *Catla catla*. Six casein-gelatin based (33% crude protein; 16.72 kJ/g gross energy) amino acid test diets with graded levels of threonine (0.75, 1.00, 1.25, 1.50, 1.75 and 2.00% dry diet) were fed to satiation to triplicate groups of fish. Analyzed threonine concentrations were 0.74, 0.96, 1.21, 1.48, 1.72 and 1.93% dry diet. Absolute weight gain (g/fish), feed conversion ratio, protein retention efficiency%, threonine deposition, RNA/DNA ratio and carcass protein significantly improved with the increase in dietary threonine and peaked at 1.48% of the dry diet. Hematological indices were also found to be best in fish fed at 1.48% threonine diet. Quadratic regression analysis of absolute weight gain, feed conversion ratio, protein retention efficiency%, threonine deposition, RNA/DNA ratio, carcass protein, hemoglobin (g/dl), hematocrit (%) and RBCs

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(10^6/mm^3) at 95% of maximum and minimum response against varying levels of dietary threonine exhibited the requirement of fingerling *C. catla* between 1.35-1.48% dry diet, corresponding to 4.09-4.48% dietary protein. Present finding would be useful in formulating threonine balanced feeds for the intensive culture of *C. catla*.

Tryptophan is an indispensable amino acid required for a wide variety of metabolic activities. Because its concentration in organisms is lowest among the all amino acids, it can easily play a rate-limiting role in protein synthesis. Moreover, it serves as the precursor of serotonin, melatonin, tryptamine, NAD, and NADP, as well as meeting the majority of the requirement for nicotinic acid. A 12-week feeding trial was conducted to evaluate the dietary tryptophan requirement of fingerling *Catla catla*. Six casein-gelatin based amino acid test diets (33% crude protein; 16.72 kJ/g gross energy) containing graded levels of L-tryptophan (0.10, 0.14, 0.19, 0.23, 0.28 and 0.34% dry diet) were fed to fish. Absolute weight gain, feed conversion ratio, protein gain, RNA/DNA ratio, hepatosomatic index, viscerosomatic index, condition factor and hematological indices improved with the increasing levels of tryptophan from 0.10 to 0.23% of dry diet. Significantly higher carcass protein was obtained at 0.23% tryptophan of the dry diet. Exponential analysis of absolute weight gain, feed conversion ratio, protein gain and RNA/DNA ratio against dietary tryptophan levels at 95% maximum and minimum responses displayed the tryptophan requirement at 0.25, 0.23, 0.25 and 0.21% dry diet, respectively. Inclusion of dietary tryptophan in the range of 0.21-0.25% dry diet, equivalent to 0.64-0.76% dietary protein is recommended in formulating tryptophan-balanced feed for the culture of this fish species.

Histidine plays a very important role in maintaining the osmoregulation process in fishes and also a role related to energy production. It is used in metabolic pathways during certain emergencies or harsh conditions. To investigate the histidine requirement of fingerling *Catla catla*, six casein-gelatin based diets (33% CP; 13.54 kJ/g DE) containing graded levels of L-histidine (0.25, 0.39, 0.53, 0.67, 0.83 and 0.96% analyzed histidine of the dry diet) were fed near to satiation for 12 weeks. Maximum absolute weight gain (AWG; 8.63 g/fish), protein gain (PG; 1.45 g/fish), histidine gain (HG, 48.19 mg/fish), RNA/DNA ratio (4.15), best feed conversion ratio (FCR; 1.31), highest

hemoglobin (Hb, 9.61 g/dl), RBCs (2.84 x 10^6/mm^3) and hematocrit (Ht, 30.12%) were recorded in fish fed diet containing 0.67% histidine. However, quadratic regression analysis of AWG, PG, HG, RNA/DNA ratio, FCR, Hb, Ht and RBCs against dietary histidine at 95% maximum and minimum response reflected the histidine requirement at 0.63, 0.66, 0.66, 0.70, 0.67, 0.66, 0.68 and 0.63% dry diet, respectively. Carcass protein was found to improve significantly ($P<0.05$) from 13.36 to 16.42% with the increase of dietary histidine from 0.25 to 0.67%. Based on regression analysis of AWG, PG, HG, RNA/DNA ratio, FCR, Hb, Ht and RBCs, it is recommended that the diet for fingerling catla should contain histidine in the range of 0.63-0.70% dry diet, equivalent to 1.91-2.12% of the dietary protein for optimum growth, feed utilization, blood profile and carcass composition.

Methionine is one of the most limiting amino acids in many fish diets especially those containing plant protein sources such as soybean meal, peanut meal, copra meal, leucaena leaf meal, or cassava leaf meal. Methionine is an indispensable amino acid which along with the dispensable amino acid cystine constitutes the total sulfur amino acids. As cystine can only be synthesized from a methionine precursor, a portion of the methionine requirement can be spared by cystine in some fish species. So, it is important to consider the dietary cystine concentration to quantify the total sulphur amino acid requirement of the cultured species for maximum growth and efficient feed utilization. Two separate 12 weeks feeding trials were performed to quantify the total sulphur amino acid (TSAA) requirement (experiment I) and cystine replacement value for methionine (experiment II) of fingerling *Catla catla*. In experiment I, six casein-gelatin based (33% crude protein; 16.72 kJ/g gross energy) diets with graded levels of TSAA (0.56, 0.81, 1.06, 1.31, 1.56 and 1.81% dry diet) were made. The TSAA requirement was determined by quadratic regression analysis of absolute weight gain (AWG), protein efficiency ratio (PER), feed efficiency (FE), protein gain (PG) and total sulphur amino acid gain (TSAAG) against dietary TSAA concentrations at 95% maximum response. Above analysis revealed that inclusion of TSAA at 1.28% dry diet (1.22% methionine + 0.06%cystine), corresponding to 3.87% of dietary protein is optimum. In experiment II, to determine the replacement value of cystine for methionine, six diets containing 1.28%

TSAA determined in experiment I with different ratios of L-methionine and L-cystine (80:20, 70:30, 60:40, 50:50, 40:60 and 30:70) on equimolar sulphur basis were fed to fish. Quadratic regression analysis of AWG, PER, FE, PG and TSAAG against varying methionine to cystine ratios yielded the optimum cystine replacement value of about 39%. Based on above analysis it is recommended that inclusion of 1.28% dietary TSAA, corresponding to 3.87% of dietary protein is optimum of which 39% could be spared by cystine.

Phenylalanine, an aromatic indispensable amino acid is required for normal growth and metabolic processes. It is the sole precursor of tyrosine. Phenylalanine can be converted to tyrosine by tetrahydrobiopterin-dependent phenylalanine hydroxylase in liver and kidneys but phenylalanine can not be synthesized back from tyrosine. Thus, adding tyrosine to diets for fish can reduce requirement for phenylalanine. Since the requirement for phenylalanine depends on the amount of tyrosine in the diet, the total aromatic amino acid is taken into consideration in meeting the amino acid needs. Two 12 weeks experiments were conducted to determine the dietary total aromatic amino acid requirement and tyrosine replacement value for phenylalanine for fingerling *Catla catla*. In experiment I, phenylalanine requirement was determined by feeding six casein-gelatin based amino acid test diets (33% crude protein; 16.72 kJ/g gross energy) with graded levels of phenylalanine (0.39, 0.64, 0.87, 1.12, 1.38 and 1.62% dry diet) at a constant level (1%) of dietary tyrosine to triplicate groups of fish near to satiation. Absolute weight gain (AWG g/fish), feed conversion ratio (FCR), protein retention efficiency (PRE%), phenylalanine retention efficiency (PHRE%) and RNA/DNA ratio responded positively with the increasing concentrations of phenylalanine reaching the highest values at 1.12% of the dry diet. Quadratic regression analysis of AWG, PRE, PHRE and RNA/DNA ratio at 95% of maximum response against varying levels of dietary phenylalanine exhibited the requirement at 1.02, 0.99, 0.97 and 1.06% dry diet, respectively. The above analysis revealed that inclusion of phenylalanine at 1.01% of dry diet, corresponding to 3.06% dietary protein is optimum. In experiment II, six diets with different levels of L-tyrosine (0.19, 0.38, 0.59, 0.81, 0.98 and 1.18% dry diet) with 1.01% phenylalanine (determined in experiment I) fixed in all the test diets were fed to fish to

determine the tyrosine requirement under identical conditions. Quadratic regression analysis of AWG, PRE, PHRE and RNA/DNA ratio at 95% of maximum response against dietary tyrosine concentrations indicated the requirement at 0.64, 0.65, 0.71 and 0.73% dry diet, respectively. Hence, inclusion of tyrosine at 0.68% of the dry diet, corresponding to 2.06% of dietary protein is taken as the tyrosine required for optimum utilization of phenylalanine. Based on above data, total aromatic amino acid requirement of fingerling *C. catla* was found to be 1.69% (1.01% phenylalanine+0.68%tyrosine) of the dry diet, corresponding to 5.12% of dietary protein and tyrosine replacement value for phenylalanine was found to be 36.7% on molar basis. It is recommended that fingerling *C. catla* require 1.69% TAAA of the dry diet (5.12% of dietary protein) of which 37% (on molar basis) could be supplied as tyrosine.

The data generated during the present study on essential amino acid requirements of fingerling *C. catla* have been summarized below:

Essential amino acids	**Requirements**	
	%dry diet	**%dietary protein**
Arginine	1.67	5.06
Lysine	1.7-1.8	5.2-5.5
Isoleucine	1.13-1.18	3.42-3.58
Valine	1.02	3.09
Leucine	1.57-1.59	4.76-4.82
Threonine	1.35-1.48	4.09-4.48
Tryptophan	0.21-0.25	0.64-0.76
Histidine	0.63-0.70	1.91-2.12
Total sulphur amino acids	1.28 (39% on equimolar sulphur basis could be spared by cystine)	3.87
Total aromatic amino acids	1.69 (37% on molar basis could be supplied as tyrosine)	5.12

The information generated during the present study on the quantitative essential amino acid requirements of *C. catla* would be useful in formulating amino acid balanced, cost-effective practical feeds for the intensive culture of this fish.

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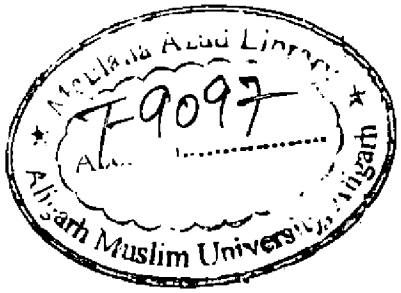

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CERTIFICATE

This is to certify that the thesis entitled **"Dietary essential amino acid requirements of Indian major carp, *Catla catla* (Hamilton) fingerling"** has been completed under my supervision by Ms. Seemab Zehra. The work is original and has been pursued by the candidate independently. It embodies some interesting observations contributing to the existing knowledge on the subject.

I permit the candidate to submit the work for the award of degree of **Doctor** of **Philosophy** in **Zoology** of the Aligarh Muslim University, Aligarh, India.

Mukhtar A. Khan

Dr. Mukhtar A. Khan
Professor

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DEDICATED TO MY PARENTS

ACKNOWLEDGMENTS

By the grace of Almighty Allah, I feel extremely happy to express my heartfelt appreciation to my supervisor, Prof. Mukhtar A. Khan, for his invaluable guidance and support during my research and compilation of this thesis. Besides providing indispensable guidance during each and every phase of my research work, he has been a constant source of inspiration to me. I am heavily indebted to him for his benevolent attitude and advice on personal and professional level which strengthened my morale, punctuality, determination and will power. I shall always remember his painstaking interest, patience and constant encouragement despite all my lapses and shortcomings.

I am highly grateful and indebted to the Chairman, Department of Zoology, Prof. Iqbal Parwez for providing necessary laboratory facilities and valuable suggestions. I sincerely appreciate his kindness and generosity. I am also thankful to Dean Faculty of Life Science, Prof. Irfan Ahmed for his constant support and encouragement. I am extremely obliged to Prof. S. M. Abbas Abidi, Prof. Wajihullah, Prof. Qudsia Tahseen, Dr. Malik Irshadullah, Dr. Mohd. Afzal Khan, Dr. M.K. Saifullah and Dr. Saltanat Parveen for their continuous encouragement.

I am immensely indebted to my lab seniors Dr. S. F. Abidi and Ms. Farhat with whom I shared my views, suggestions and some of the most thought provoking and intellectually stimulating discussions during the entire period of my research work. I am also indebted to my lab collegues Mr. M. Musharraf and Mr. Y. M. Khan for their timely assistance and cooperation during the tenure of this work.

I have been fortunate enough to get the support of my best friend Mrs. Syeda Sadaf Raza. Without the love, support and continuous encouragement of her, my dream of pursuing Ph D would have not come true. Therefore, I would like to express my special thanks to her.

I express my sincere thanks to my friends Ms. Shahnaz Parveen, Ms. Habeeba A. Kabeer, Ms. Nuzhat Parveen and Uzma Ahmad for their encouragement and loving support.

Finally I would like to express my special thanks and indebtness to my parents for their sacrifice, unconditional love and never ending care without whom this effort may not have borne fruits. I wish to acknowledge the superb assistance, affection and sustain moral support shown by my sisters and brothers. I would like to extend my profound heartfelt thanks to my younger sister Ms. Mursaleen Fatima for all her love, encouragement and endless support which undoubtedly helped me retaining my sanity even throughout the hard times of my work. Her love, care and faith on me is much appreciated.

I wish to thank University Grant Commission for granting the Maulana Azad fellowship for looking after my welfare.

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CONTENTS

GENERAL INTRODUCTION

GENERAL INTRODUCTION

The world population has increased dramatically during the twentieth century. Although agriculture production has kept pace with world population growth, the rate of population growth has outstripped the agriculture production significantly. As a result of that, there is a shortage of protein, fat and calories in the diet of people. A plausible solution of the problem is aquaculture. Aquaculture is the most dynamic and rapidly growing sector of the global agricultural economy, and it remains the leading hope for bridging the widening gap between supply and demand for fish products. One of the major constraints in aquaculture is the unavailability of nutritionally balanced feeds. Proper nutrition is one of the most important factors influencing the ability of cultured organism to attain the genetic potential for growth, reproduction and longevity (De Silva and Anderson 1995), and its status is considered as one of the important factors that determine the ability of fish to resist diseases. If the feed is not consumed by the fish or if the fish are unable to utilize the feed because of some nutrient deficiency, then there will be no growth. An undernourished animal can not maintain its health and be productive, regardless of the quality of its environment (Bureau and Cho 1999). Nature offers a great diversity of food to fish including plants and animals. Artificial feed plays an important role in semi intensive fish culture where it is required to maintain a high density of fish than the natural fertility of the water can support (Jhingran 1991). The role of artificial feed in intensive fish farming cannot be ignored as nutritional requirements of fish depend upon the feed supplied (Rahman and Mustafa 1989a,b; Srivastava et al. 2013). The quantity and quality of feed consumed have an impactful effect on growth, feed conversion and proximate composition of fish (Mustafa et al. 1997; Jena et al. 1998). Supplementary feeding is known to increase the carrying capacity of culture systems and can enhance fish production by many folds (Devaraj et al. 1976).

The commercial viability of fish culture depends on market demand and cost of production. The largest fraction of the production cost (40-70%) lies in the fish feed (Webber and Huguenin 1979; Cho et al. 1985; De Silva 1989; Ogbe et al. 2004; Otubusin et al. 2007). Therefore, formulation of cost-effective feeds can significantly influence the profitability. One of the challenges is to develop less wasteful, cost effective feeds and

development of such feeds is dependent on knowing a species nutritional requirements and meeting those requirements with balanced feed formulations and appropriate feeding practices (Gatlin III 2010; Ahmed et al. 2011). Although there is an increasing evidence that gross nutrient requirement of cultured species group are close to each other, but the requirements may vary with other factors like fish size, age, temperature and nutrient balanced in diet, etc. (Kim et al. 1992; Akiyama et al. 1997; Ruchimat et al. 1997; Forster and Ogata 1998; Simmons et al. 1999; De Silva et al. 2000; Zhou et al. 2007; Ahmed 2009). The percentage requirements of these components vary from species to species and are also age-specific within the same species. Therefore, knowledge on nutrition and practical feeding of fish is essential. Practical diets for fish are formulated to contain all nutrients and sufficient energy for satisfactory growth and proper health. To express their maximum growth potential, animals require balanced diet that meet their nutritional needs at each stage of development (Sa et al. 2006). A formula for balanced fish diets must include an energy source plus sufficient indispensable amino acids, essential fatty acids, specific vitamins and minerals to support life and to promote adequate growth (Halver 1976; 2002; NRC 2011).

Fish require three macronutrients, protein, fat and carbohydrate, along with many substances and elements classified as micronutrients. Among these macronutrients, protein is one of the most expensive components of fish feed. It is usually given most attention as it is considered the main nutrient affecting fish growth (NRC 2011; Zehra and Khan 2012; Kpogue et al. 2013). Although insufficient dietary protein does not allow fish to express their maximum growth potential, excess dietary protein is not desirable, both from an economical and an environmental point of view. Indeed, if protein is included in excess in the diets, prices increase unnecessarily and the excess of protein is catabolized, leading to an increase of nitrogen load to the environment. Thus, it is important to formulate diets that meet, but do not exceed protein requirements for maximum growth, while minimizing feed costs and water pollution (Sa et al. 2006). Referring to protein requirements is nevertheless usual in fish nutrition as protein includes both indispensable amino acids and dispensable amino acids that provide the undifferentiated nitrogen required for the synthesis of nitrogenous compounds of

physiological interest (Oliva-Teles 2012). From a quantitative point of view, efficiency of protein utilization and muscle protein growth are the most critical issues. Optimizing the amino acid supply in tune with the requirements and improving protein utilization for body protein growth with limited impacts on the environment in terms of nutrient loads is a generic imperative in all aquaculture production system (Kaushik and Seiliez 2010). Amino acids and their metabolites are important regulators of key metabolic pathways that are necessary for maintenance, growth, feed intake, nutrient utilization, immunity, behavior, larval metamorphosis, reproduction as well as resistance to environmental stressors and pathogenic organisms in various fishes (Li et al. 2009). Amino acids that cannot be synthesized by the animals or are not synthesized in sufficient amounts to meet physiological requirements and must be supplied in the diets are referred to as essential or indispensable amino acids. For those that can be synthesized in adequate quantity are termed non-essential or dispensable amino acids (Lim and Webster 2006). These amino acids have nutritional significance because their presence in the diet conserves energy that would be required for synthesis, and some dispensable amino acids can partly replace or spare indispensable amino acids, for example methionine and phenylalanine are required as specific precursors for the synthesis of the dispensable amino acids cystine and tyrosine, respectively. Some amino acids are readily converted to glucose to provide an essential energy source for some critical body organs and tissues such as brain and red blood cells. Since carbohydrate is not prevalent in their natural diet, fish are more dependent upon amino acids as precursors to glucose than most other animals. Therefore, a portion of the dietary protein or amino acids is always used as an energy source in fish. Some of the amino acids can cause reduced performance by animals through amino acid antagonism. When these amino acids are fed in excess of their required levels, they cause an increase in the requirement for other structurally similar amino acids (Lovell 1998). A well-described antagonism between lysine and arginine and among branched chain amino acids has been reported in some fish species (Berge et al. 1999; Alam et al. 2002; Ahmed and Khan 2006; NRC 2011). Kaushik and Fauconneau (1984) recorded the reduced plasma arginine at higher levels of dietary lysine in rainbow trout due to lysine arginine interaction. Nutritional and metabolic interactions among the branched-chain amino acids isoleucine, leucine and valine have been reported for various warm blooded animals,

including man (Hambraeus et al. 1976); poultry (De'Mello and Lewis 1971; Smith and Austic 1978); rat (Harper et al. 1970); and the pig (Oestemer et al. 1973). Data on interactions among branched-chain amino acids in fish are not clear-cut and are inconsistent among species (Yamamoto et al. 2004). Chance et al. (1964) reported that isoleucine requirement in chinook salmon was influenced by dietary leucine and that excess dietary isoleucine reduced growth rates when leucine was deficient. Certain amino acids, when offered to animals in excessive amounts, can produce adverse reactions that range in severity from reduced growth and feed intake to the occurrence of pathological lesions and death (Alam et al. 2002).

All fish species studies to date have been shown to require ten indispensable amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine) in their diet for maximum growth (Wilson 1985). Growth of fish is primarily based on the muscle protein deposition (Carter and Houlihan 2001) which is due to the flow of amino acids from feed to protein synthesis sites (Ronnestad et al. 2003). If any of the essential amino acid is not present in sufficient amount or present in excessive amounts relative to other amino acids, protein synthesis will not be supported (Hepher 1988; Abidi and Khan 2007). Dietary amino acid imbalances can lead to increased deamination and oxidation of those amino acids not contributing to protein synthesis and consequently increased nitrogenous excretion (Cho and Kaushik 1985). It has been widely acknowledged that feeding diets with amino acid deficiencies results in altered protein deposition and excess energy deposition as fat in the liver, fillet or peritoneal cavity (Gaylord and Barrows 2009). Since the essential amino acids must be obtained from the diet, an overabundance or deficiency of any of the essential amino acids may have severe pathological consequences. Lack of quantified requirements of individual amino acids for the particular species poses challenges for emerging aquaculture aquafeeds (Farhat and Khan 2012a). It is necessary to obtain the knowledge of the specific amino acid requirements of the fish species and to prepare mixtures of protein supplemented with the deficient amino acids and thus achieve a maximum growth and protein efficiency.

Development of first successful amino acid test diet for chinook salmon by Halver (1957) enabled a number of workers to study the essentiality of various amino acids in other fish species (Halver and Shanks 1960; Shanks et al. 1962; Nose et al. 1974; Mazid et al. 1978; Nose 1979). Later studies on the quantitative amino acid requirement were mostly based on the test diet in which the nitrogen component consisted either of crystalline amino acid or a mixture of amino acids with selected whole protein source, commonly casein and gelatin (Halver 2002; NRC 2011; Pohlenz et al. 2012; Ren et al. 2013; Farhat and Khan 2012a, b; 2013a,b), zein (Dabrowski 1981) or gluten (Halver et al. 1958; Ketola 1983; Zhou et al. 2012). The amino acid profile of the total protein component of the diet being controlled so as to simulate the amino acid pattern of the specific reference protein. To assure the maximum utilization of the amino acids such diet contains protein at slightly below the optimum requirement. Quantitative amino acid requirements of some fish have been studied using crystalline amino acids as the only nitrogen sources. Crystalline amino acids are highly soluble and thus losses through leaching are a major impediment to their use in fish feeds. In addition to this, free amino acid are absorbed faster and assimilated. It has been argued that rapid amino acid absorption rates may lead to amino acid imbalances, increasing amino acids catabolism instead of protein synthesis (Zhang 2007). Therefore, when crystalline amino acids are supplied in the diet, it is necessary to coat them to delay the passage time and absorption of amino acids making them available for protein synthesis (Alam et al. 2004). Hence, use of casein and gelatin in combination with a mixture of crystalline L-amino acids often take advantages over the use of crystalline amino acids only and thus, have usually been used to quantify amino acid requirements in many aquatic animal species (NRC 2011; Pohlenz et al. 2012; Ren et al. 2013).

Dose-response experiments generally are used to determine amino acid requirements in fish (Cowey 1992; Rodehutscord et al. 1997; Rodehutscord and Pack 1999). Ideally the only ingredient to vary within such experiments should be the amino acid being investigated, while all other substances should be kept constant. The quantitative requirements for ten essential amino acids using dose-response approach have been demonstrated in a number of cultivable fish species examined so far (NRC

2011). Amino acid requirements have also been reported for black sea bream *Acanthopagrus schlegelii* (Zhou et al. 2011a,b); rohu *Labeo rohita* (Abidi and Khan 2011); channel catfish *Ictalurus punctatus* (Pohlenz et al. 2012); stinging catfish *Heteropneustes fossilis* (Khan and Abidi 2011a,b; Farhat and Khan 2012a,b; 2013a,b; Ahmed 2012a,b); yellow croaker *Pseudosciaena crocea* (Xie et al. 2012); largemouth bass *Micropterus salmoides* (Zhao et al. 2012a); Jian carp *Cyprinus carpio* (Zhao et al. 2012b,c; Dong et al. 2013); yellow catfish *Tachysurus fulvidraco* (Cao et al. 2012), mrigal *Cirrhinus mrigala* (Khan and Abidi 2013); Chinese sucker *Myxocyprinus asiaticus* (Lin et al. 2013); Nile tilapia *Oreochromis niloticus* (Ovi and Eze 2013); blunt snout bream, *Megalobrama amblycephala* (Ren et al. 2013); red sea bream *Chrysophrys major* (Rahimnejad and Lee 2013); golden pompano *Trachinotus ovatus* (Niu et al. 2013) and Jian carp, *Cyprinus carpio* (Tang et al. 2013).

Cyprinids form quantitatively the most important group of freshwater teleost fishes cultured around the world. Among these, the Indian major carps (IMCs), catla, *Catla catla*, rohu, *L. rohita* and mrigal, *C. mrigala* are fast-growing fishes attaining marketable size of 800-1000 g in less than a year, and are generally propagated on extensive and/or intensive scale in polyculture systems (Jhingran and Pullin 1988). The Indian major carps have a large number of advantages over other fish species. These carps have lower production costs, fewer environmental problems and a smaller risk of disease outbreaks compared to other species of fish (ADB/NACA 1996; Abidi and Khan 2008). Catla is endemic to the riverine system in northern India, Indus plain and adjoining hills of Pakistan, Bangladesh, Nepal and Myanmar, and has been introduced later into almost all riverine systems, reservoirs and tanks all over India. The natural distribution of catla seems to be governed by temperature dependency rather than latitude and longitude. It is one of the most preferred food fish. It is mostly cultivated in isolated freshwater ponds and lakes in the absence of carnivorous fish. The fish is fleshy and noted for its delicacy and valued very high in the market. It is best for consumption when not more than 61cm in length (FAO 2006). The Indian major carp, *C. catla,* is a promising species for aquaculture exploitation with its rapid growth and good market potential. In terms of value-added, processed fish products, this species should have

potential as the present market price of this fish is ranging between Rs. 80 to 140 per kilogram in Indian markets (Srivastava et al. 2013). Catla, along with the other Indian major carps, also form the mainstay of culture practices, contributing approximately 5.4 million tonnes to the total aquaculture production in 2010 (FAO 2012). India also dominates the global Catla production by contributing about 71% to the global total of 3.87 million tonnes (FAO 2010). Its highest growth potential, coupled with high consumer preference, has established Catla as an important freshwater species for aquaculture. It has been known that the successful intensive fish farming depends on the development of low-cost but nutritionally efficient artificial diets and the improvement of production technology. Currently, the lack of data on nutrient requirements of this fish is one of the major constraints for developing low-cost and nutritionally rich diet for this fish. Information on some nutritional aspects of *C. catla* are available (Renukaradhya and Varghese 1986; Singh and Bhanot 1988; Khan and Jafri 1991; Ravi and Devaraj 1991; Nandeesha et al. 1991; 2001; Sinha and Sinha 1994; Seenappa and Devaraj 1995; Biswas et al. 1996; Swain et al. 1999; Nandi et al. 2001; Khan and Abidi 2008; Sukumaran et al. 2009; Dars et al. 2010; Murugesan et al. 2010; Kumar et al. 2011; Kaleeswaran et al. 2011; Abidi and Khan 2012 and Srivastava et al. 2013).

Although essential amino acid requirements of the fry *C. catla* have been reported by Ravi and Devaraj (1991), no published data on amino acid requirements of the fingerling stage of this fish is available. The present study was, therefore, undertaken to generate data on all ten essential amino acid requirements of fingerling *C. catla* and the findings are presented in the form of this thesis.

GENERAL METHODOLOGY

GENERAL METHODOLOGY

Source of fish stock and their acclimatization

Induced bred fry *C. catla* were procured from G. B. Pant University of Agriculture and Technology, Pantnagar. These were transported to the wet laboratory in oxygen filled polythene bags, given the prophylactic dip in $KMnO_4$ solution (1:3000), and stocked in indoor circular aluminium plastic lined (Plastic crafts corpn, Mumbai, India, 1.22 m in diameter; 0.91 m in height) rearing tanks (water volume 600 L) for a fortnight. During this period, the fish were fed casein-gelatin based (33% Crude protein) H-440 diet (Halver 2002) and reared to fingerling stage.

Feeding trials

Fish of the desired size and number were sorted out from the acclimatized fish lots maintained in the wet laboratory. These were stocked in triplicate groups in 70L high-density polyvinyl circular troughs (water volume 55L) fitted with continuous water flow-through system. The water exchange rate in each trough was maintained at 1.0-1.5 L/min. After carefully observing the feeding behavior of the fish, they were fed six days a week to apparent satiation thrice a day at 08:00, 12:30 and 17:30h. The feeding trials lasted for twelve weeks. Initial and weekly body weights were recorded on a top loading balance (Precisa 120A; 0.1 mg sensitivity, Oerlikon, AG, Zurich, Switzerland). Troughs were siphoned off to remove faecal matters before feeding, daily. Accumulation of the diet at the bottom of the trough was avoided. Uneaten food was siphoned off immediately, dried in a hot air oven and reweighed to measure the amount of food consumed. On the day of weekly measurements, fish were fasted for 24 h to empty their guts and their mass weight recorded on a top-loading balance (Precisa 120A; 0.1 mg sensitivity, Oerlikon AG, Zurich, Switzerland) for calculating other growth parameters. Troughs were scrubbed and disinfected thoroughly with water and $KMnO_4$ solution on the day they were batch weighed. Mortality, if any, was recorded. At the end of the experiment, desired number of fish were randomly sacrificed and kept in freezer (-20°C) for the assessment of carcass composition.

Preparation of experimental diets

To work out the dietary essential amino acid requirement of fingerling *C. catla,* amino acids test diets (33% crude protein; 16.72 kJ/g gross energy) with graded levels of test amino acids were formulated. The dietary range necessary to quantify the requirement of test amino acids was adjusted on the basis of existing information on other carps (NRC 2011). Dietary protein level was fixed at 33%, which is lower than the optimum protein requirement of fingerling *C. catla* (35%, Khan and Jafri 1991; Dars et al. 2010). This reduction was done to assure maximum utilization of the limiting amino acids (Wilson 2002). Crystalline L-amino acids, excluding the test amino acids, were used to simulate the amino acid profile of the experimental diets to that of 33% whole chicken egg protein. The amount of test amino acids was added to the experimental diets at the expense of glycine on protein to protein basis to make the desired dietary concentrations of test amino acids. Diets were made isonitrogenous and isoenergetic by adjusting the levels of glycine, and the dextrin in all the experiments conducted for estimating the essential amino acid requirements of fingerling *C. catla.* A combination of cod liver oil and corn oil (2:5) was used as a source of lipid. Vitamin and mineral premixes were prepared as per Halver (2002). Pre-weighed quantities of crystalline L-amino acids and salt mixture were thoroughly stirred in hot water (80°C) in a steel bowl attached to a Hobart electric mixer (K5SS, Hobart Corp., Troy, Ohio, U.S.A.). The pH of the resulting mixture was adjusted to neutral with 6 N NaOH solution (Nose et al. 1974). Crystalline L-amino acids (CAA) were coated with some amount (5% of the diet) of cooked carboxymethyl cellulose (CMC). Gelatin was dissolved separately in a volume of water with constant heating and stirring and then transferred to the CMC-bound pre-coated CAA mixtures. These pre-coated CAA mixtures were further coated with cooked casein at 80°C. The mixer bowl was removed from heating and dextrin added. Vitamin and oil premixes, were added to the lukewarm bowl (50°C) one by one with constant mixing. Lastly, rest 5% carboxymethyl cellulose was added to the above mixture and the speed of the blender was gradually increased as the diet started to harden. The dough was passed through a pelletizer fitted with a 2-mm die to obtain pellets which were dried in a hot air oven at 40°C to reduce the moisture content below 10%. The dry pellets thus obtained were

crumbled, sieved (500 μm) and stored at 4°C until used. The coating of crystalline L-amino acids with carboxymethyl cellulose followed by casein and gelatin provided sufficient water stability. In addition to providing sufficient water stability, coating of crystalline L-amino acids also reduces the absorption rate of the amino acids (Cho et al. 1992) and leaching (Alam et al. 2004). We adopted this approach as a measure to prevent the leaching of crystalline amino acids from the test diets and optimize their use for protein gain. To determine the leaching loss of the amino acids from the test diets, the dried samples after immersion in water for 30 min were also subjected to amino acid analysis. The amino acid analysis of these dietary samples exhibited no significant change in their amino acid composition.

Estimation of water stability of the diets

Water stability of the diet was estimated by the method of Fagbenro and Jauncey (1995). Briefly, representative samples (5 g) of amino acid test diets were placed on a sieve and slowly immersed in a 70-litre experimental tanks containing deionized water (water volume 55 l) at 27°C for 10 min. The sieves were removed and the crumbles allowed to drain for 1 min, oven-dried at 105°C for 2 h, cooled in a desiccators and reweighed. The water stability of the amino acid test diets in all experiments were calculated which was found to range between 96-98%.

Proximate analyses

Assessment of proximate composition of ingredients, diets and body were made using standard techniques (AOAC 1995).

Moisture

A known quantity of sample was taken in a pre-weighed crucible and placed in a hot air oven at 105±1°C for 24 hours. After complete drying, the sample was cooled at room temperature in desiccators and was reweighed. The loss in weight gave an index of water from which its percentage was calculated.

Fat

Crude fat was estimated by continuous soxhlet extraction technique (Socs Plus, SCS 4, Pelican equipments, Chennai, India) using petroleum ether (40-60°C B.P.) as solvent. Finely powdered and dried sample (2-4 g) was placed in fat extraction thimble and placed in a clean, dry pre-weighed beaker to which 80 ml petroleum ether was added. This beaker was then placed in the soxhlet assembly for the extraction of fat for 2-3 hours. After extraction the beaker was removed and kept in hot air oven (100°C) to evaporate the traces of solvent. It was then transferred to a desiccator, cooled and reweighed. The difference between the weight of the beaker before and after gave the quantity of crude fat extracted from the unknown amount of the sample. The result was expressed as percentage on dry weight basis.

Crude protein

The estimation of crude protein (N x 6.25) was done using an auto Kjeldahl system (Kjeltec Foss Tecator$^{2300\ TM}$, Hoganas, Sweden) after acid digestion with an auto-digester (Foss, Tecator, Hoganas, Sweden). A known quantity of sample was taken in Kjeltec digestion tubes. To this, 0.8 g of copper sulphate, 7.0 g potassium sulphate and 12 ml. of concentrated sulphuric acid were added. The content was digested in pre-heated (420°C) digestion block of the instrument. The process of digestion continued for 60 minutes until clear blue/green solution was obtained. Now the digested sample was cooled at room temperature and titrated automatically in distillation unit of the instrument. The level of protein displayed on the screen was noted down.

Ash

A known quantity of dried powdered sample (2-5 g) was taken in pre-weighed silica crucible and incinerated in oven incineration at 650°C using muffle furnace (S. M. Scientific Instrument (P) ltd. Jindal Company, New Delhi, India) for 2-4 h or till the sample became carbon-free and completely white. The crucible was cooled in desiccators and reweighed to estimate the quantity of ash. The result was expressed as percentage on dry weight basis.

Gross and digestible energy

Gross energy was determined on a ballistic bomb calorimeter (Gallenkamp, Loughborough, England). Prior to estimate, a known quantity of dried powdered sample (0.5-1.0 g) was taken in metallic crucible and compacted carefully to increase the rate of combustion at 25 lb oxygen pressure. The heat generated upon combustion was read on the modulated galvanometer scale, and converted to energy equivalent, worked out earlier using the thermo chemical grade benzoic acid (26.42 kJ/g) as a standard. The gross energy was expressed as kJ/g. Energy of ingredients used in the test diets was calculated as 23.08, 20.199, 16.02, 24.27 and 37.64 kJ/g for casein, gelatin, dextrin, amino acid and fat, respectively as estimated on Gallenkamp ballistic bomb calorimeter. Digestible energy was calculated on the basis of physiological fuel values 18.83, 14.64 and 35.56 kJ/g for protein, carbohydrate and fat, respectively (Jauncey 1982).

Amino acid analysis

Amino acid analysis of casein, gelatin, experimental diets, initial and final fish carcass was carried out with the help of Hitachi L-8800 Amino Acid Analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan) by hydrolyzing 0.3 mg sample in 1 mL of 6 N HCl for about 22 h. The sample thus obtained was diluted in 0.02 N HCl. The hydrolyzed samples were filtered using microfilter (Cellulose acetate membrane, 0.45 µm, Corning, Japan) and then injected in an automatic Amino Acid Analyzer (Hitachi L-8800). Recovery hydrolysis of tryptophan was performed in 4 N-methanesulfonic acid instead of 6 N HCl followed by the decomposition at 110°C for 22 h. After this, 4 N NaOH was added to adjust the pH to approximately 2. This was then diluted again in 0.02 N HCl. However, the recovery hydrolysis of sulphur amino acids methionine and cystine was performed in 2 mL of performic acid for 4-24 h. After this, 0.3 mL of 48% HBr was added, and the decomposition was performed at 110°C for 22 h. The samples were then dried solid under reduced pressure. After this, 1 mL of 0.2 N NaOH was added, and sample was then left stand still for about an hour. Lastly, the pH and volume of the sample was adjusted using 0.05 N HCl and 0.1 N HCl.

RNA and DNA estimation

RNA and DNA were determined by the method of Schneider (1957). At the termination of feeding trial, three fish from each replicate of the treatment group (n=3x3) were euthanized with an overdose of MS-222 and white muscle was removed. Three subsamples of the muscle samples for each replicate of the treatment group were taken for the determination of RNA and DNA. Muscle samples were homogenized for 5 min in 5% trichloroacetic acid (TCA) at 90°C and then centrifuged at 5000 g for 20 min. For the determination of RNA, 2.0 ml of distilled water and 3.0 ml of orcinol reagent was added in 1.0 ml of supernatant. The reaction mixture was kept in boiling water bath for 20 min. The greenish-blue colour thus developed was read at 660 nm in a spectrophotometer (Genesis 10-UV, Thermo Spectronic, Madison, USA). For DNA determination, 1.0 ml of distilled water and 4.0 ml of freshly prepared diphenylamine reagent were added to 1.0 ml of the supernatant. The reaction mixture was kept on a boiling water bath for 10 min. The blue colour developed was measured at 600 nm. Standard curves for RNA and DNA were drawn using different concentrations of yeast RNA and calf thymus DNA, respectively. The values were expressed as µg/100 mg fish muscle tissue on dry basis.

Evaluation of growth performance

Growth performance of the *C. catla* in response to increasing levels of dietary amino acids was measured by calculating the following parameters:

Absolute weight gain (g/fish) = Final body weight-Initial body weight

Feed conversion ratio = Dry feed intake/Wet weight gain

Feed intake (g/fish) = Total dry feed intake (g)/Total no. of fish

Protein efficiency ratio = Weight gain/Protein intake

Feed efficiency = Wet weight gain/Dry feed intake

Nutrient retention efficiency% = Nutrient gain/Nutrient intake x 100

Nutrient gain (mg/fish) = Final body nutrient content x final body weight-initial body nutrient content x initial body weight x 1000

Nutrient deposition = Nutrient gain/Nutrient intake

Hepatosomatic index% = Liver weight (g)/Body weight (g) x 100

Viscerosomatic index% = Viscera weight (g)/Body weight (g) x 100

Condition factor = Body weight (g)/Total body length $(cm)^3$ x 100

Survival Rate (%) = Final number of fingerling collected/Initial number of fingerling stocked x 100

Statistical analyses

Responses of fingerling *C. catla* fed graded levels of test amino acids were subjected to one-way analysis of variance (Sokal and Rohlf 1981). Differences among treatment means were determined by Tukey's HSD test at a $P<0.05$ level of significance. Dietary amino acid requirements of fingerling *C. catla* were estimated by fitting the quadratic (Zeitoun et al. 1976; Shearer 2000) and exponential (Rodehutscord et al. 1997) regression analyses to the dose-growth responses relationship. The amino acid requirements for maximum growth performance are defined as the point on the abscissa representing 95% of the value of the upper asymptote on the ordinate (Rodehutscord et al. 1997; Dias et al. 2003; Gaylord et al. 2005). The equations employed for quadratic and exponential analyses were $Y=aX^2+bX+c$, $Y=a\ (1-exp^{-k(x-xc)})$ and $y = a+b*exp^{-kx}$, respectively. All the statistical analyses were done using Origin (version 6.1; Origin Software, San Clemente, CA).

Table 1 Composition of the mineral mixture*

Minerals	g/100 g of mineral mixture
Calcium phosphate (Dibasic)	13.57
Calcium lactate	32.69
Ferric citrate	2.97
Magnesium sulphate	13.20
Potassium phosphate (Dibasic)	23.98
Sodium phosphate (Monobasic)	8.72
Sodium chloride	4.35
Aluminium chloride.$6H_2O$	0.015
Potassium iodide	0.015
Cuprous chloride	0.010
Manganous sulphate. H_2O	0.080
Cobalt chloride.$6H_2O$	0.100
Zinc sulphate	0.300

*Halver 2002

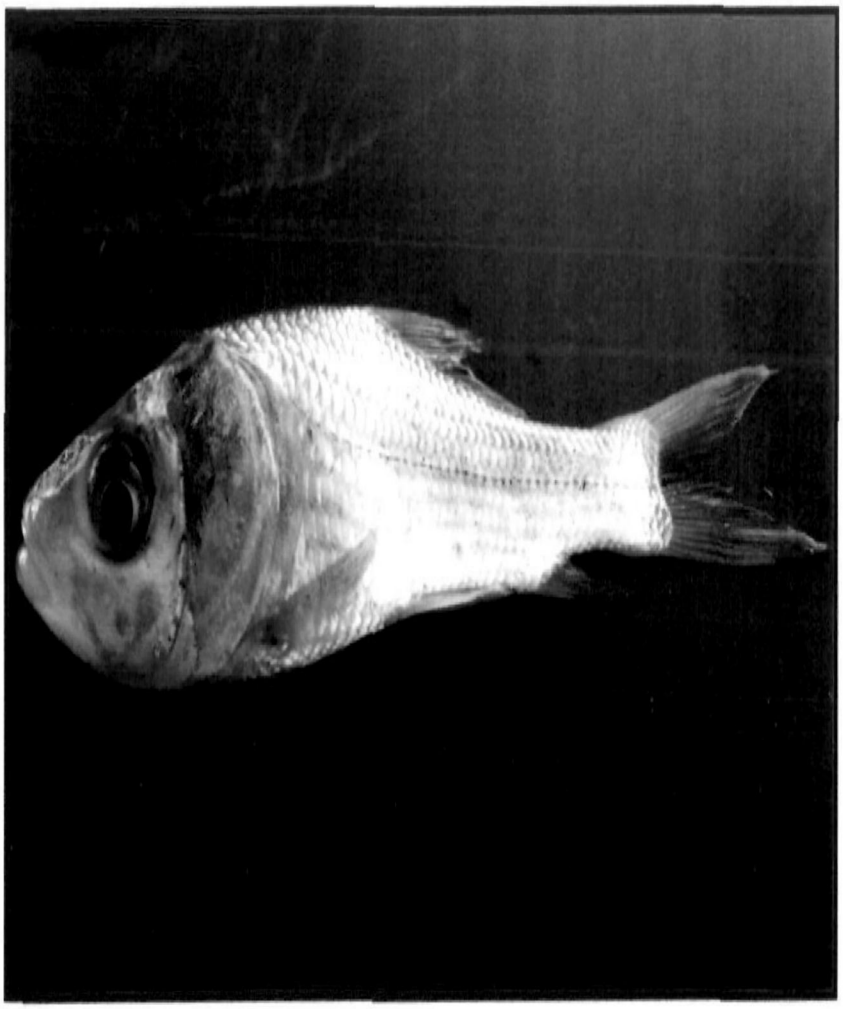
Table 2 Composition of the vitamin mixture*

Vitamins	g/100 g dry diet
Alpha cellulose	2.000
Choline chloride	0.500
Inositol	0.200
Ascorbic acid	0.100
Niacin	0.075
Calcium pantothenate	0.050
Riboflavin	0.020
Menadione	0.004
Pyridoxine hydrochloride	0.005
Thiamine hydrochloride	0.005
Folic acid	0.0015
Biotin	0.0005
Alpha tocopherol acetate**	0.040
Vitamin B_{12}***	0.00001(0.5 ml)

*Halver 2002

**Incorporated with oil

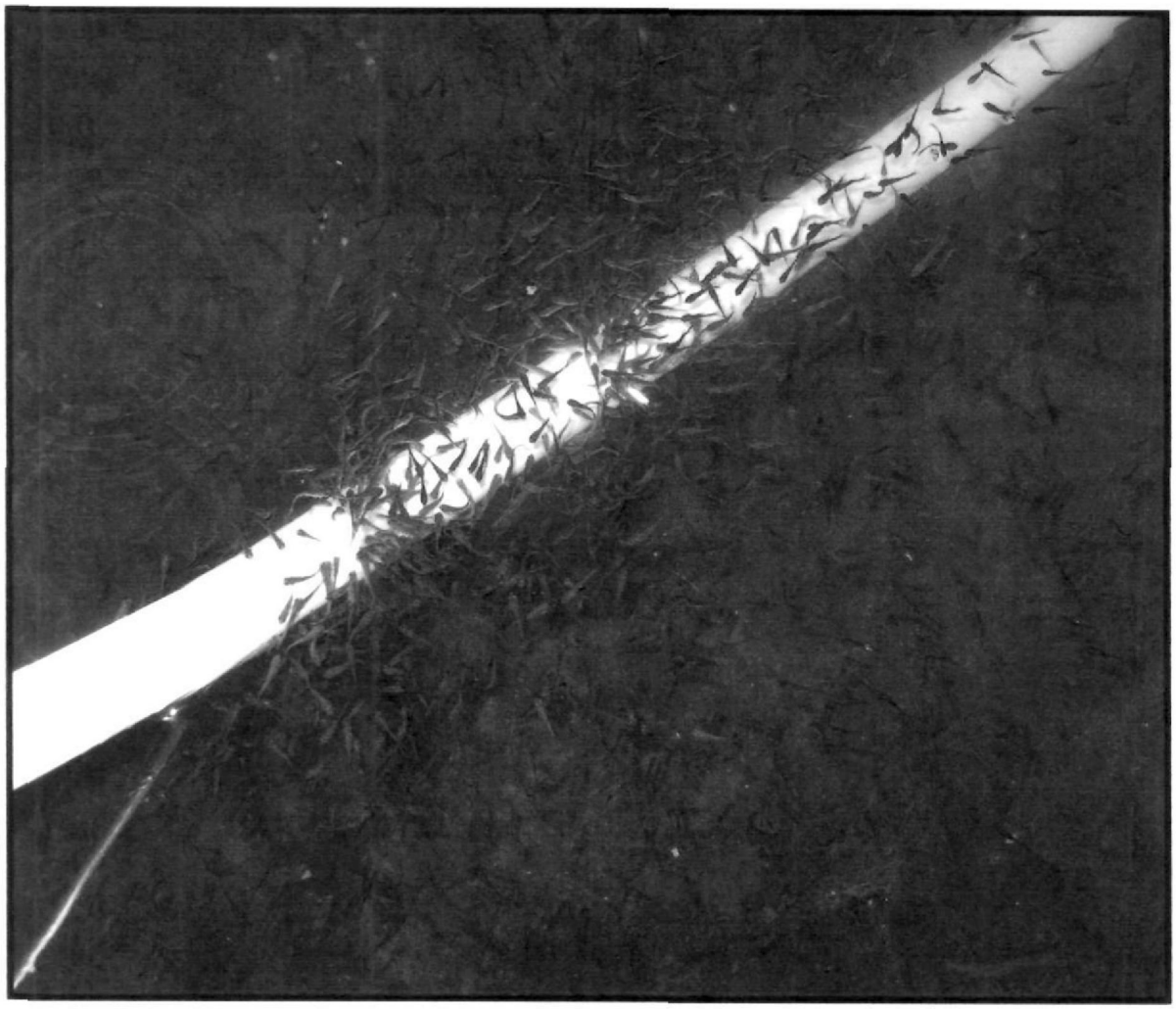
*** (10 mg/500 ml H_2O)



Catla catla (Hamilton, 1822)

Kingdom	Animalia
Phylum	Chordata
Subphylum	Vertebrata
Super class	Gnathostomata
Series	Pisces
Class	Teleostomi
Subclass	Actinopterygii
Order	Cypriniformes
Family	Cyprinidae
Genus	*Catla*
Species	*catla*

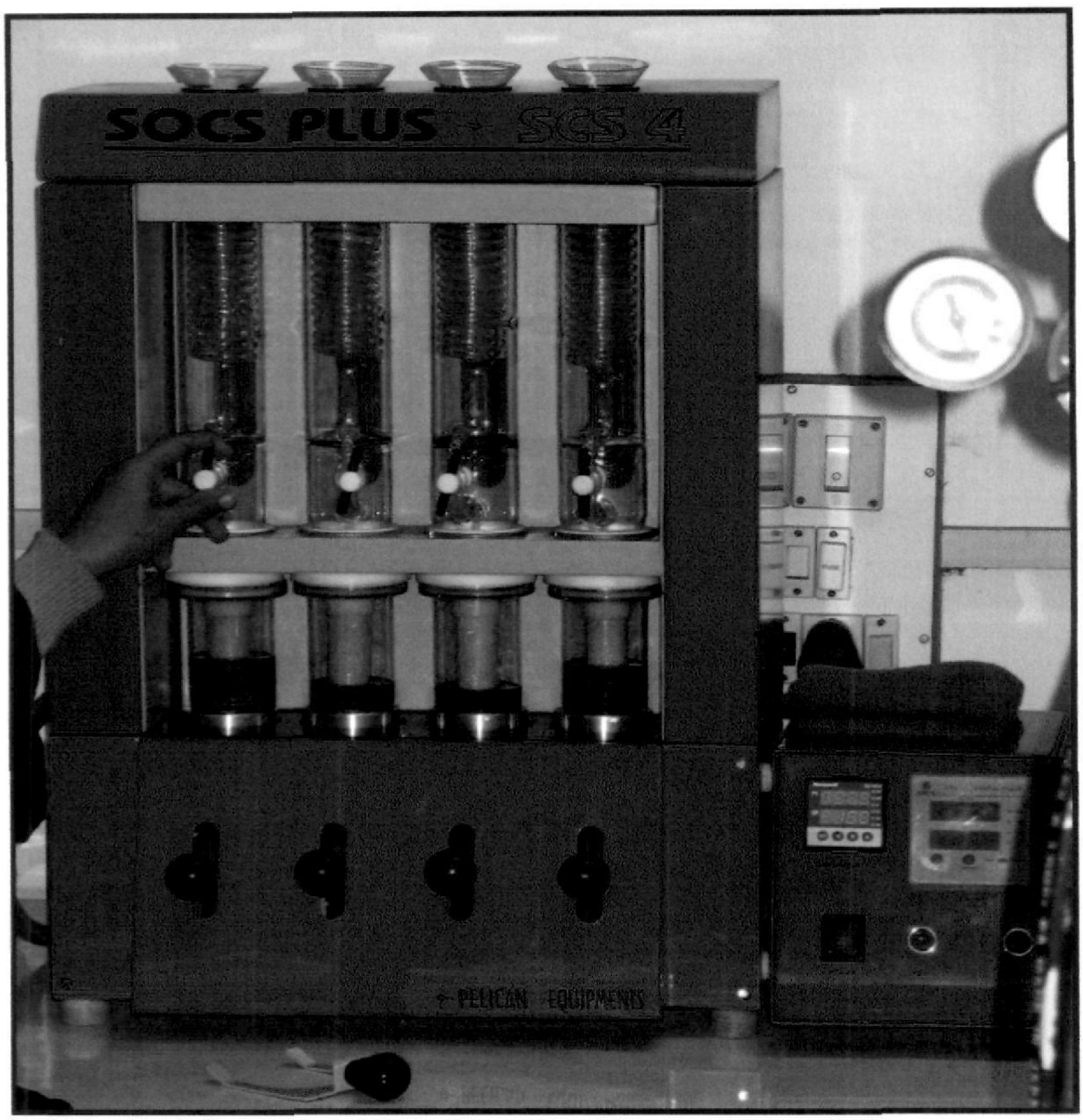
Rearing Tanks



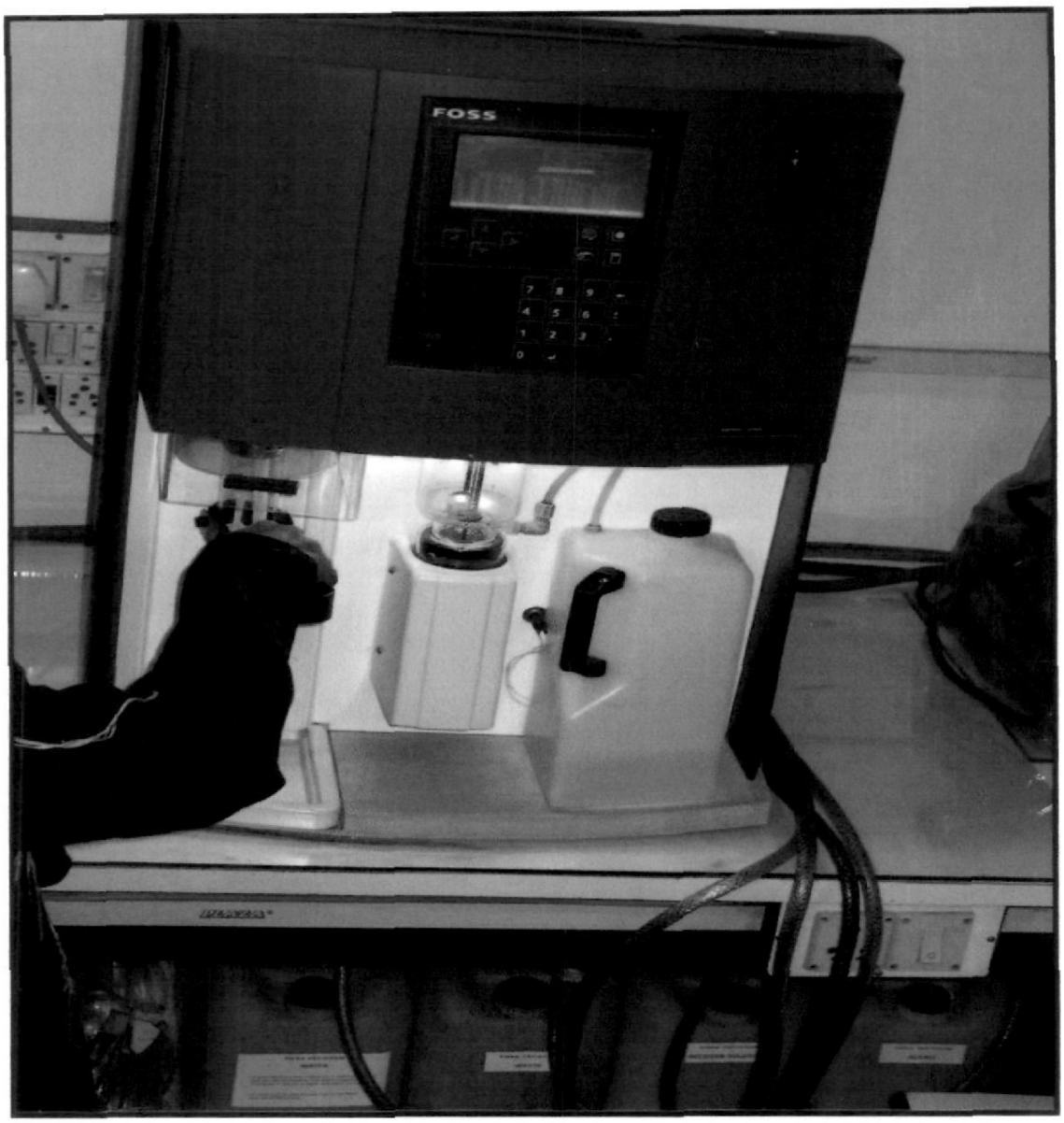
Experimental Fish

Flow-Through System

Gross Energy Estimation Equipment (Gallenkamp Ballistic Bomb Calorimeter)

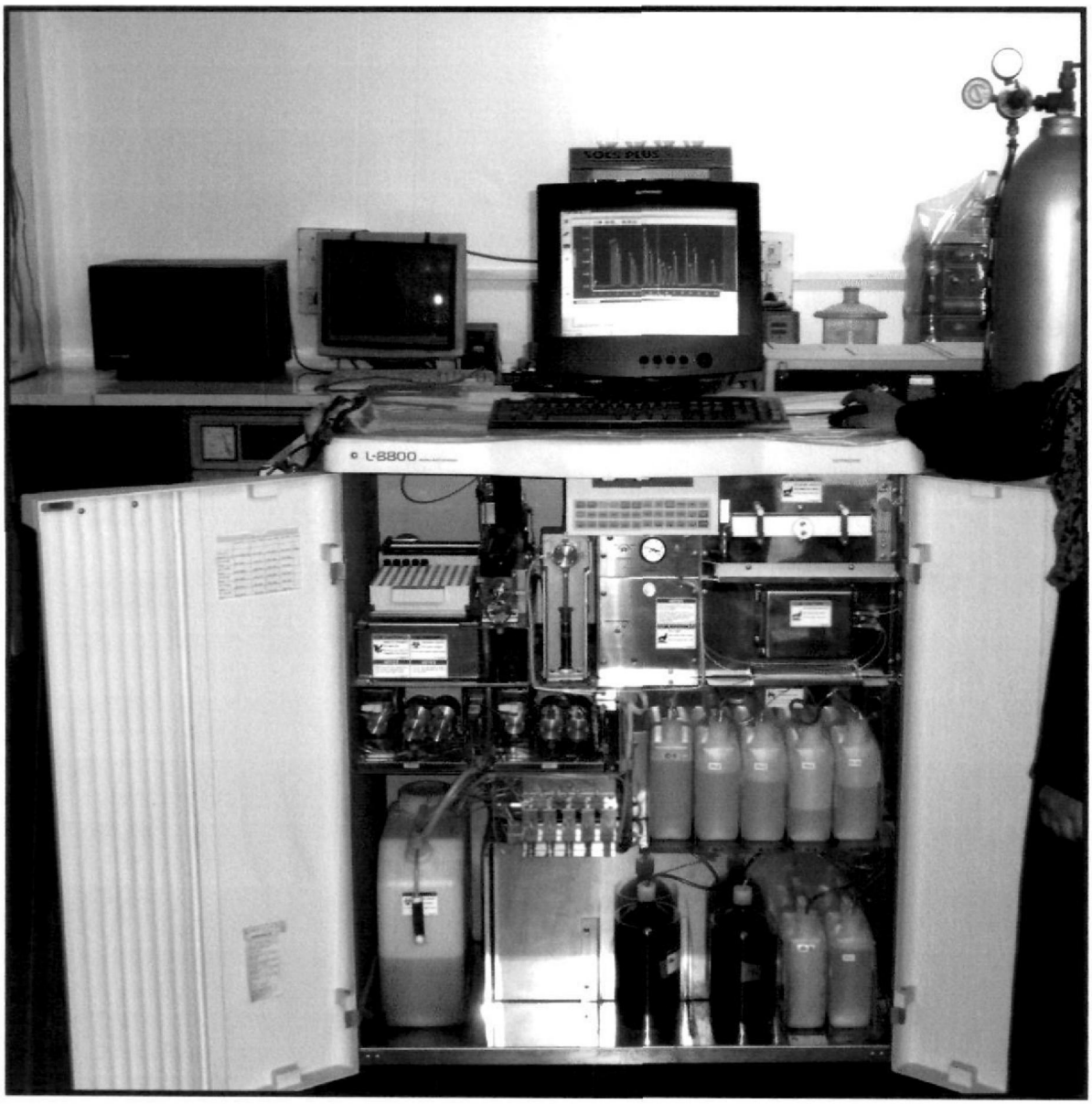


Fat Extraction Equipment (Socs Plus)



Nitrogen Estimation Equipment (Kjeltec Tecator)

Ash Estimation Equipment (Muffle Furnace)



Amino Acid Analyzer (Hitachi L-8800)

CHAPTER 1

CHAPTER 1

DIETARY ARGININE REQUIREMENT OF FINGERLING INDIAN MAJOR CARP, *CATLA CATLA* (HAMILTON)*

INTRODUCTION

In intensive culture systems nutritionally complete feeds have been used and constitute a major proportion of aquaculture production cost. Hence, the development of cost-effective feeds is critical to economic success of aquaculture system. Protein and amino acids are costliest components of fish feed (Nguyen and Davis 2009). Fish do not require protein *per se*, rather they require amino acids that comprise protein. Amino acids are not only important substrates for the synthesis of proteins and other nitrogenous compounds but also act as key regulators of fluxes through major metabolic pathways (Jobgen et al. 2006). Dietary deficiency of any essential amino acid will impair protein synthesis and suppress fish growth (Masagounder et al. 2010). Additionally, feeding excess concentrations of essential amino acid can result in increased ammonia excretion and degrade water quality (Hart et al. 2010). Therefore, to provide appropriate amounts of these nutrients in feeds, precise information on essential amino acids requirement of cultured species is crucial.

Arginine has important nutritional and physiological roles and is established as indispensable in the diet of many fish species (NRC 2011). In addition to as an important component of protein, arginine is involved in several biologically important metabolic pathways. It is a precursor of at least six biologically important compounds and thus is one of the most metabolically versatile amino acid (Morris 2006). Arginine affects metabolism of proteins, amino acids, glucose, fatty acids and, therefore, the development (Flynn et al. 2002). It is an essential component of the urea cycle which is the major pathway for elimination of ammonia in mammals (Flynn et al. 2002; Kohli 2003). In freshwater teleost, the activity of the urea cycle is very low compared with mammals (Depeche et al. 1979) and hence the essentiality of arginine would be more pronounced in fish than in growing mammals. The presence of a full urea cycle in teleostean fish

*Published in '*Journal of the World Aquaculture Society*' 44: 363-373 (2013)

(Huggins et al. 1969) including rainbow trout (Chiu et al. 1988) suggests a potential for arginine biosynthesis. All species require substantial amounts of dietary arginine for maximum growth. This indicates that although an animal possesses the enzyme necessary for arginine synthesis, the amount of arginine synthesized may not be enough to meet the need for maximum growth.

Dietary arginine requirements have been worked out for many fish species such as Japanese flounder, *Paralichthys olivaceus* (Alam et al. 2002); mrigal, *Cirrhinus mrigala* (Ahmed and Khan 2004a); hybrid Clarias, *Clarias gariepinus* x *C. macrocephalus* (Singh and Khan 2007); rohu, *Labeo rohita* (Abidi and Khan 2009); black sea bream, *Acanthopagrus schlegelii* (Zhou et al. 2011a) and stinging catfish, *Heteropneustes fossilis* (Khan and Abidi 2011a). Information on arginine requirement of fry *Catla catla* is available (Ravi and Devaraj 1991). However, dietary arginine requirement of fingerling stage of this fish is completely lacking. As the arginine requirement reported by Ravi and Devaraj (1991) was based on weight gain only which may also be owing to the deposition of fat or moisture content, it could not be an accurate parameter for nutrient requirement studies. Therefore, in addition to weight gain, the present work reports the arginine requirement of fingerling based on sensitive parameters such as feed conversion, protein deposition, arginine retention, RNA/DNA ratio and carcass composition of fingerling *C. catla*.

MATERIALS AND METHODS

Experimental diets

Six isonitrogenous (33% crude protein) and isocaloric (16.72 kJ/g gross energy) amino acid test diets with graded levels of arginine (1, 1.25, 1.5, 1.75, 2 and 2.25% dry diet) were prepared using casein (vitamin and fat-free), gelatin and crystalline L-amino acids (Table 1). The diets were designated as A_1, $A_{1.25}$, $A_{1.75}$, A_2 and $A_{2.25}$. The levels of arginine in the amino acid test diets were fixed on the basis of information available on other carps (Ahmed and Khan 2004a; Abidi and Khan 2009; NRC 2011). The amino acid compositions of the experimental diets, excluding the test amino acid arginine, were

simulated to that of 33% whole chicken egg protein. The arginine content contributed by the casein and gelatin was 0.54 and 0.41% of the dry diet, respectively. To make the intended concentrations of dietary arginine in the amino acid test diets, the amount of arginine was increased at the expense of glycine on protein basis. Method of preparation of experimental diets has been discussed under the General Methodology section (pages 9-10).

Experimental design and feeding trial

Source of the fish, their acclimation and details of the general experimental design has already been discussed under the General Methodology section (page 8).

Fingerling *C. catla* (3.55±0.05 cm; 0.61±0.02 g) were taken from the above acclimated fish lot and stocked in triplicate groups in 70 L circular polyvinyl troughs (water volume 55 L) fitted with a continuous water flow-through (1-1.5 L/min) system at the rate of 25 fish per trough for each dietary treatment level. Fish were fed test diets in the form of dry crumbles (500 µm) to apparent satiation thrice daily at 08:00, 12:30 and 17:30h. Initial and weekly weights were recorded on a top-loading balance (Precisa 120A; 0.1 mg sensitivity, Oerlikon AG, Zurich, Switzerland) after anaesthetizing the fish with tricaine methane sulfonate (MS-222; 100 µg/ml). Fish were deprived of feed on the day they were weighed. The feeding trial lasted for 12 weeks. Faecal matter was siphoned before every feeding. Water quality parameters were monitored daily during the feeding trial and were recorded following standard methods (APHA 1992). The range of water temperature, dissolved oxygen, free carbon dioxide, pH, total ammonia nitrogen, nitrites and total alkalinity based on daily measurements, was 27.2-28.6°C, 6.8-7.3 mg/L, 5.5-9.3 mg/L, 7.3-7.6, 0.31-0.35 mg/L, 0.04-0.08 mg/L and 65.4-82.6 mg/L, respectively.

Chemical analyses

Proximate composition of casein, gelatin, experimental diets, and initial and final carcass was estimated using standard methods (pages 10-11). Gross energy content was determined on a Gallenkamp Ballistic Bomb Calorimeter as per the method described on page 12. Amino acid analysis of casein, gelatin, experimental diets (Table 2), initial and

final fish carcass was done using an automatic amino acid analyzer as detailed earlier (page 12). At the beginning of the feeding trial, 60 fish were randomly sampled, killed and pooled together. Six subsamples of a pooled sample were analyzed for initial carcass composition. At the end of the experiment, 20 fishes from each replicate of dietary treatments were randomly collected, sacrificed with an overdose of MS-222 and pooled separately. Three subsamples of the pooled samples were analyzed for final carcass composition.

Determination of RNA and DNA

RNA and DNA were determined by the method of Schneider (1957) as described earlier on page 13.

Evaluation of growth parameters

Calculation of various growth parameters was made according to the standard definitions as described under the General Methodology section (pages 13-14).

Statistical analyses

Statistical analyses of growth data were done using the procedures as detailed earlier (page 14).

RESULTS

Data for absolute weight gain (AWG; g/fish), protein efficiency ratio (PER), feed conversion ratio (FCR), protein deposition (PD), arginine retention efficiency (ARE%) and feed intake are illustrated in Table 3. These parameters were affected by varying concentrations of dietary arginine. Fish receiving 1.75% dietary arginine ($A_{1.75}$) reflected best AWG (6.93 g/fish), PER (2.20) and FCR (1.42). Similarly highest PD (0.36) and ARE (78%) were recorded at this level. Feed intake remained almost constant among the treatments. Fish fed diets containing more than 1.75% dietary arginine (A_2-$A_{2.25}$) exhibited reduced growth in terms of weight gain, feed conversion, protein deposition and arginine retention. Fish fed diet with 1% arginine (A_1) showed poorest AWG (2.77

g/fish), PER (0.84), FCR (3.71), PD (0.11) and ARE (38%). Survival in all the treatments was found to be 100% irrespective of the dietary arginine levels.

In order to generate more precise data on arginine requirement of fingerling *C. catla*, all the growth data were subjected to quadratic regression analysis at 95% of maximum or minimum response. Quadratic regression analysis of AWG (Fig. 1), FCR, PER, PD and ARE against dietary arginine concentrations exhibited the optimum arginine requirement of fingerling *C. catla* between 1.64-1.69% of the dry diet.

Carcass composition of fingerling *C. catla* was also affected quadratically in response to varying levels of arginine in the diets excepting carcass ash content (Table 4). Carcass protein content in fish fed diet containing 1.75% arginine ($A_{1.75}$) was higher compared to the groups receiving other dietary arginine concentrations at 1 (A_1), 1.25 ($A_{1.25}$), 1.5 ($A_{1.5}$), 2 (A_2) and 2.25% ($A_{2.25}$). The quadratic relationship of carcass protein to dietary arginine is given in Table 5. According to this model, at 95% of the asymptote of carcass protein the calculated optimal arginine requirement was found to be 1.65% of the dry diet. Carcass fat decreased linearly (4.68-2.84%) with the increase in dietary arginine levels from 1-2.25% (A_1-$A_{2.25}$). Moisture content was found to be negatively correlated with the carcass fat. Ash content remained almost unchanged among varying test groups. The DNA content of the white muscle sample was found to decrease from 1-1.75% of the dietary arginine (A_1-$A_{1.75}$). However, the muscle RNA and RNA/DNA ratio improved with the increased inclusion of dietary arginine up to 1.75% (Table 4). The data for RNA/DNA ratio were also subjected to quadratic regression analysis which at 95% of maximum response exhibited the arginine requirement at 1.66% of the dry diet.

On the basis of above results, the arginine requirement of fingerling *C. catla* was found to be 1.67% of the dry diet which is taken as a mean of arginine requirement reflected by several response variables. The equations employed for each response variables have been summarized in Table 5.

DISCUSSION

During the present study, maximum growth was noted in fish fed diet containing 1.75%

arginine ($A_{1.75}$). However, fish fed diets containing more than above level (2-2.25%) of dietary arginine (A_2-$A_{2.25}$) reflected reduction in growth. Similar growth depressing effect in fish fed higher doses of arginine was also recorded in other fish species such as mrigal (Ahmed and Khan 2004a); hybrid clarias (Singh and Khan 2007) and rohu (Abidi and Khan 2009). This growth depression may be due to the stress caused by excess amount of amino acids in the body of the fish leading to extra energy expenditure towards deamination and excretion of the same (Walton 1985). The reduction in growth at higher levels of dietary arginine could also be attributed to amino acid toxicity. The accumulation of an amino acid or its degradative products in body pools may stress enzymatic systems and lead to further accumulation and possible toxicity (Alam et al. 2003) which may have an adverse effect on growth because disproportionate intake affects absorption and utilization of other amino acids or decreases the diet's palatability (Borlongan and Coloso 1993). Also, it has been reported that the major proportion of dietary limiting amino acids is used for protein synthesis while amino acids in excess will be more available for oxidation (Gahl et al. 1996) which may be the reason for growth depression at higher levels of dietary arginine.

Dietary arginine supplementation can beneficially increase protein gain and reduce carcass fat accretion (Tan et al. 2009). Carcass protein content was found to be maximum in fish fed diet containing 1.75% arginine ($A_{1.75}$). Further inclusion of dietary arginine showed reduction in carcass protein content indicating that the amount of arginine exceeding this level might have not been used for increasing body protein synthesis. Arginine supplementation increases lipolysis and inhibits lipogenesis by modulating the expression and function of key enzymes which are involved in anti-oxidative response and fat metabolism in insulin-sensitive tissues (Jobgen et al. 2009). In this study, carcass fat content responded negatively with the increase in dietary arginine concentrations. The decrease in carcass fat with the increasing concentrations of dietary arginine, as evident in this study, was also reported by Khan and Abidi (2011a) in stinging catfish. Arginine also plays a crucial role in regulating extra-endocrine signaling pathways such as AMP-activated protein kinase pathway (Yao et al. 2008) which has inhibitory effects on biosynthetic pathways for fatty acid and sterol synthesis (Viollet et

al. 2003; Zhou et al. 2011a) leading to linear reduction in carcass fat in this study.

The RNA/DNA ratio is considered to be a useful and reliable indicator of fish growth. The quantity of DNA in an animal cell is believed to be normally stable but the quantity of RNA is closely related to the rate of body protein synthesis (Tanaka et al. 2007). Since protein production varies in accordance with the quantity of RNA, the RNA/DNA ratio has been used to evaluate growth of many fishes (Mustafa 1977; Mustafa and Mittal 1982; Mustafa and Zofair 1985; Bulow 1987; Mustafa et al. 1991; Peck et al. 2003; Mercaldo-Allen et al. 2008). The RNA/DNA ratio in fingerling *C. catla* exhibited a quadratic response pattern to increasing concentrations of dietary arginine and improved up to 1.75% arginine of the dry diet ($Arg_{1.75}$). Further increment in dietary arginine (Arg_2-$Arg_{2.25}$) showed a decline in RNA/DNA ratio. Almost similar response was also reported by Abidi and Khan (2009) in *L. rohita.*

Since protein deposition and amino acid retention reflect true picture of an amino acid requirement, these parameters were also used in this study to work out the arginine requirement of this fish. The quadratic regression analysis at 95% of maximum response of growth parameters indicated the optimum arginine requirement of fingerling *C. catla* to be at 1.67% of the dry diet. This requirement is higher than that reported for channel catfish, *Ictalurus punctatus* 1.2% (NRC 2011); rainbow trout, *Oncorhynchus mykiss* 1.5% (NRC 2011); rohu 1.22-1.39% (Abidi and Khan 2009) and lower than that reported for catla 1.92% (Ravi and Devaraj 1991), Japanese flounder 2.25% (Alam et al. 2002); mrigal 1.8% (Ahmed and Khan 2004a); coho salmon, *O. kisutch* 2.2% (NRC 2011); black sea bream 2.8-3.1% (Zhou et al. 2011a) and approximately comparable to common carp, *Cyprinus carpio* 1.7% (Nose 1979), Atlantic salmon, *Salmo salar* 1.6% (Lall et al. 1994) and yellow perch, *Perca flavescens* 1.6% of the dry diet (Twibell and Brown 1997). It has been suggested that the wide variability and the reliability of arginine requirements of fish may be affected by fish size and age, feeding regime, feed allowance, adequate levels of other nutrients, water temperature, flow rate, stock density, environmental and culture conditions adopted in different laboratories (Chiu et al. 1988; Luzzana et al. 1998; Hansen et al. 2010). Response criteria and the mathematical model used to estimate the optimal value may also affect the arginine requirements of fish (Zhou

et al. 2011a).

The arginine requirement of fingerling *C. catla* worked out during this study (1.67% of the dry diet) is lower than the requirement reported by Ravi and Devaraj (1991) for fry stage of this fish (1.92% of the dry diet). Higher amino acid requirement estimates are associated with the smaller size fish whereas it becomes comparatively lower with the advancing stages because smaller size fish have higher rate of metabolism compared with larger size (Conklin 2000). This may probably be the reason for the differences in the arginine requirement as fish under study was in fingerling stage requiring lower dietary nutrient requirements for the metabolical and physiological activities than the fry stage of the fish in the study conducted by Ravi and Devaraj (1991). Also, they adopted restricted feeding strategy in their experiments whereas in present study, satiation feeding at three feeding frequencies was adopted to ensure the maximum feed intake. Most importantly, Ravi and Devaraj (1991) have used the crystalline L-amino acids in uncoated form which hamper the amino acid utilization by lowering its gut retentivity (Murai et al. 1982; 1984) whereas in present study, the crystalline L-amino acids were coated with casein and gelatin that promoted the retention time of the amino acids in the gut leading to more efficient utilization of the ingested amino acids. These reasons may considerably affect the arginine requirement of *C. catla.*

Except for poor growth and feed utilization efficiency, no arginine related deficiency signs were observed during the entire length of the feeding trial. Absence of any arginine deficiency signs in the present study indicates that the diet containing minimum level of arginine (1%) was sufficient to prevent the pathological signs in this fish.

Based on quadratic regression analysis of growth parameters against dietary arginine concentrations, optimum arginine requirement of fingerling *C. catla* is recommended to be 1.67% of the dry diet. Data generated during the present study would be useful to formulate arginine-balanced feeds for mass culture of this fish.

SUMMARY

Dietary arginine requirement of fingerling *Catla catla* (3.55±0.05 cm; 0.61±0.02 g) was determined by feeding casein-gelatin based isonitrogenous (33% crude protein) and isocaloric (16.72 kJ/g gross energy) amino acid test diets containing six graded levels of L-arginine (1%, 1.25%, 1.5%, 1.75%, 2% and 2.25% dry diet) for 12 weeks. Maximum absolute weight gain (AWG; 6.93 g/fish), protein efficiency ratio (PER; 2.13), protein deposition (PD; 0.36), arginine retention efficiency (ARE; 78%) and best feed conversion ratio (FCR; 1.42) were recorded in fish fed 1.75% arginine of the dry diet. Maximum carcass protein (15.57%) and RNA/DNA ratio (4.79) were also recorded for the group fed 1.75% arginine of the dry diet. Quadratic regression analysis at 95% maximum or minimum response of above growth parameters yielded the arginine requirement of fingerling *C. catla* at 1.67% of the dry diet. On the basis of above analysis of the growth parameters, it is recommended that inclusion of dietary arginine at 1.67% of the dry diet, corresponding to 5.06% dietary protein is optimum for formulating arginine-balanced, cost-effective quality feeds for the mass culture of fingerling *C. catla*.

Table 1 Composition of the experimental diets for fingerling *C. catla* (IBW[1]=0.61 g/fish) for 12 weeks

	Dietary arginine levels (%)					
Ingredients (g/100 g dry diet)	**1.00 (A_1)**	**1.25 ($A_{1.25}$)**	**1.50 ($A_{1.5}$)**	**1.75 ($A_{1.75}$)**	**2.00 (A_2)**	**2.25 ($A_{2.25}$)**
Casein[a] (fat-free)	15	15	15	15	15	15
Gelatin[b]	5	5	5	5	5	5
Dextrin	32.27	32.55	32.82	33.10	33.37	33.65
Amino acid mixture[c]	18.32	18.14	17.96	17.78	17.60	17.42
Corn oil	5	5	5	5	5	5
Cod liver oil	2	2	2	2	2	2
Mineral mix[d,f]	4	4	4	4	4	4
Vitamin mix[e,f]	3	3	3	3	3	3
α- Cellulose	5.41	5.31	5.22	5.13	5.03	4.94
Carboxymethyl cellulose	10	10	10	10	10	10
Total	100	100	100	100	100	100
Calculated gross energy[g] (kJ/g, dry diet)	16.72	16.72	16.72	16.72	16.72	16.72
Estimated gross energy (kJ/g, dry diet)	16.48	16.51	16.63	16.72	16.68	16.32
Digestible energy[h] (kJ/g, dry diet)	14.19	14.20	14.17	14.21	14.21	14.23

[a]Crude Protein (76%); Loba Chemie, Mumbai, India; [b]Crude Protein (96%); Loba Chemie, Mumbai, India; [c]Amino acid mixture (g/100 g dry diet) arginine variable, histidine 0.26, isoleucine 1.747, leucine 1.44, lysine 0.953, methionine 0.789, cystine 0.73, phenylalanine 1.24, tyrosine 0.67, threonine 0.768, tryptophan 0.38, valine 1.313. alanine 0.94, proline 0.527, glycine variable; (Loba Chemie, Mumbai, India); [d]Mineral mixture (g/100 g of mineral mix) calcium biphosphate 13.57; calcium lactate 32.69; ferric citrate 02.97; magnesium sulphate 13.20; potassium phosphate (dibasic) 23.98; sodium biphosphate 08.72; sodium chloride 04.35; aluminium chloride. $6H_2O$ 0.0154; potassium iodide 0.015; cuprous chloride 0.010; manganous sulphate. H_2O 0.080; cobalt chloride. $6H_2O$ 0.100; zinc sulphate. $7H_2O$ 0.40; [e]Vitamin mixture (g/100 g dry diet) choline chloride 0.500; inositol 0.200; ascorbic acid 0.100; niacin 0.075; calcium pantothenate 0.05; riboflavin 0.02; menadione 0.004; pyridoxine hydrochloride 0.005; thiamin hydrochloride 0.005; folic acid 0.0015; biotin 0.0005; alpha-tocopherol 0.04; vitamin B_{12} 0.00001; 2 g α-cellulose; [f]Halver (2002); Loba Chemie, Mumbai, India; [g]Calculated on the basis of fuel values 23.07, 20.19, 16.01, 24.28 and 37.62 kJ/g for casein, gelatin, dextrin, amino acid and fat, respectively, as estimated on Gallenkamp ballistic bomb calorimeter-CBB 330 010L (Gallenkamp, Loughbrough, U K); [h]Digestible energy was calculated on the basis of physiological fuel values 18.83, 14.64 and 35.56 kJ/g for protein, carbohydrate and fat, respectively (Jauncey 1982).

Table 2 Analyzed amino acid composition of the experimental diets (%) for fingerling *C. catla* (IBW[1]=0.61 g/fish) for 12 weeks[a]

	Dietary arginine levels (%)					
Amino acid	**1.00 (A_1)**	**1.25 ($A_{1.25}$)**	**1.50 ($A_{1.5}$)**	**1.75 ($A_{1.75}$)**	**2.00 (A_2)**	**2.25 ($A_{2.25}$)**
EAAs						
Arginine	0.98	1.23	1.47	1.72	1.97	2.23
Histidine	0.70	0.71	0.69	0.70	0.72	0.69
Isoleucine	2.66	2.65	2.65	2.66	2.68	2.67
Leucine	3.11	3.13	3.10	3.12	3.11	3.12
Lysine	2.42	2.40	2.39	2.41	2.40	2.38
Methionine	1.37	1.35	1.38	1.37	1.36	1.37
Phenylalanine	2.12	2.13	2.11	2.14	2.10	2.11
Threonine	1.47	1.45	1.45	1.44	1.46	1.47
Tryptophan	0.53	0.52	0.53	0.53	0.51	0.52
Valine	2.43	2.44	2.46	2.41	2.42	2.44
NEAAs						
Cystine	0.81	0.83	0.80	0.82	0.81	0.83
Tyrosine	1.51	1.52	1.53	1.50	1.51	1.52
Alanine	1.89	1.91	1.88	1.92	1.87	1.88
Aspartic acid	1.16	1.15	1.14	1.13	1.16	1.17
Glycine	7.91	7.51	7.09	6.65	6.21	5.78
Proline	2.81	2.83	2.80	2.82	2.83	2.84
Serine	0.55	0.56	0.54	0.52	0.55	0.54

[a]Determined by Automatic Amino Acid Analyzer (Hitachi L-8800, Tokyo, Japan).

Table 3 Growth, conversion efficiency, protein deposition and arginine retention of fingerling *C. catla* (IBW[1]=0.61 g/fish) fed diets containing varying levels of arginine for 12 weeks[a,b]

	Dietary arginine levels (%)						
	1.00 (A_1)	1.25 ($A_{1.25}$)	1.50 ($A_{1.5}$)	1.75 ($A_{1.75}$)	2.00 (A_2)	2.25 ($A_{2.25}$)	Quadratic Pr>F[c]
Average initial weight (g)	0.58±0.02	0.61±0.03	0.60±0.05	0.59±0.02	0.61±0.08	0.61±0.08	0.507[b]
Average final weight (g)	3.35±0.07	5.27±0.07	6.49±0.14	7.32±0.09	7.27±0.13	6.49±0.13	0.0001
Absolute weight gain (g/fish)	2.77±0.04	4.66±0.06	5.89±0.05	6.73±0.07	6.66±0.02	5.88±0.10	0.0001
Feed intake (g/fish)	10.28±0.08	10.95±0.11	10.07±0.13	9.56±0.14	10.72±0.13	10.99±0.09	0.540[b]
Feed conversion ratio	3.71±0.03	2.35±0.04	1.71±0.02	1.42±0.02	1.61±0.02	1.87±0.02	0.001
Protein efficiency ratio	0.82±0.01	1.29±0.03	1.77±0.13	2.13±0.12	1.88±0.02	1.62±0.06	0.007
Protein deposition	0.11±0.01	0.19±0.01	0.27±0.02	0.34±0.02	0.28±0.01	0.23±0.01	0.014
Arginine retention efficiency%	38±1.3	49±1.1	57±2.4	78±1.6	71±1.9	62±1.2	0.049

[a]Mean values of 3 replicates ± SEM.

[b]Not statistically significant (P>0.05).

[c]Significance probability associated with the F statistic.

Table 4 Carcass composition (%wet basis) and RNA/DNA ratio of fingerling *C. catla* (IBW[1]=0.61 g/fish) fed diets containing varying levels of arginine for 12 weeks[a,b]

	Dietary arginine levels (%)							
	Initial	**1.00 (A_1)**	**1.25 ($A_{1.25}$)**	**1.50 ($A_{1.5}$)**	**1.75 ($A_{1.75}$)**	**2.00 (A_2)**	**2.25 ($A_{2.25}$)**	**Quadratic Pr>F[d]**
Moisture (%)	79.12±0.58	75.27±0.52	76.14±0.41	76.62±0.46	77.24±0.59	77.69± 0.51	78.15± 0.42	0.0001
Protein (%)	12.47±0.16	12.49±0.06	13.54±0.13	14.68±0.07	15.57±0.11	14.63±0.12	13.78±0.08	0.017
Fat (%)	2.82±0.11	4.68± 0.08	4.11± 0.04	3.55± 0.03	3.01±0.03	2.98± 0.02	2.84± 0.05	0.001
Ash (%)	2.16±0.08	2.13±0.02	2.11± 0.01	2.12±0.02	2.10±0.02	2.11± 0.03	2.12± 0.02	0.239[b]
RNA[c]	622±4.35	597±4.12	838±5.24	921±6.11	1184±6.12	1102±5.86	996±4.71	0.02
DNA[c]	426±2.41	433±2.54	312±2.92	277±3.83	247±1.63	264±2.73	269±2.54	0.008
RNA/DNA ratio	1.46±0.04	1.38±0.02	2.69±0.03	3.32±0.05	4.79±0.07	4.18±0.04	3.71±0.02	0.018

[a]Mean values of 3 replicates ± SEM.

[b]Not statistically significant (P>0.05).

[c](µg RNA or DNA/100g; dry weight basis)

[d]Significance probability associated with the F statistic.

Table 5 Quadratic equations for growth parameters of fingerling *C. catla* (initial body weight=0.61±0.02 g/fish) fed diets containing varying levels of arginine for 12 weeks

Coefficient of the quadratic equations ($Y=aX^2+bX+c$) and optimum level of arginine at 95% of maximum or minimum response					
Growth parameters	**a**	**b**	**c**	**Arg requirement[a]**	**R^2**
AWG[b]	-5.36	19.78	-11.44032	X_{opt}=1.66%	0.998
FCR[c]	3.26286	-11.9425	12.3075	X_{opt}=1.69%	0.986
PER[d]	-1.80571	6.59771	-4.03057	X_{opt}=1.64%	0.963
PD[e]	-0.30264	1.10237	-0.69897	X_{opt}=1.66%	0.969
ARE%[f]	-48.7143	172.257	-91.6628	X_{opt}=1.64%	0.866
RNA/DNA ratio	-3.96	14.8803	-9.657	X_{opt}=1.66%	0.930
Carcass protein	-3.76	14.29857	-1.85357	X_{opt}=1.65%	0.967

[a]Requirement as % of dry diet

[b]Absolute weight gain

[c]Feed conversion ratio

[d]Protein efficiency ratio

[e]Protein deposition

[f]Arginine retention efficiency

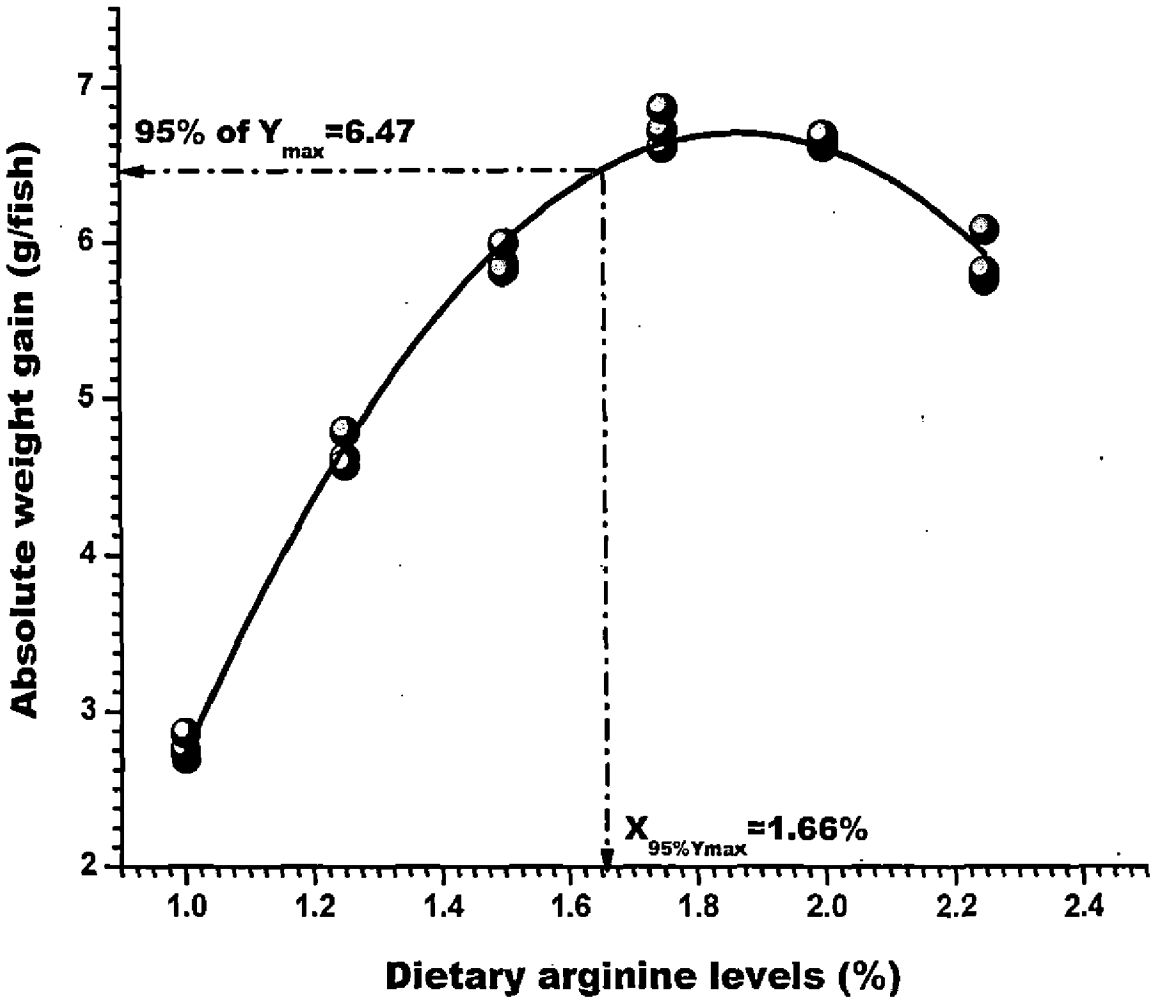


Fig. 1 Quadratic relationship of absolute weight gain to dietary arginine concentrations

CHAPTER 2

CHAPTER 2

DIETARY LYSINE REQUIREMENT OF FINGERLING *CATLA CATLA* (HAMILTON) BASED ON GROWTH, PROTEIN RETENTION, LYSINE RETENTION EFFICIENCY, RNA/DNA RATIO AND CARCASS COMPOSITION*

INTRODUCTION

Fish have quantitative requirements for each essential amino acid. Since amino acids play important and versatile roles in protein metabolism (Wright and Fyhn 2001), dietary inclusion of amino acids should be optimum to achieve maximum growth and health benefits. Of the essential amino acids, lysine is one of the most limiting essential amino acids in ingredients used for production of commercial fish feeds (Forster and Ogata 1998, Ovi and Eze 2013). Lysine is an essential amino acid present in high proportion in fish muscle tissue, involved in growth and maintenance of positive nitrogen balance, also used in "cross-linking" protein, especially collagen (UNM 2006). Information on dietary lysine requirements of cyprinids such as mrigal, *Cirrhinus mrigala* (Ahmed and Khan 2004b); grass carp *Ctenopharyngodon idella* (Wang et al. 2005); rohu, *Labeo rohita* (Abidi and Khan 2010a); common carp, *Cyprinus carpio* (Zhou et al. 2008) and other cultivable fish species (NRC 2011) are available.

Although information on lysine requirement of the fry stage of *C. catla* is available (Ravi and Devaraj 1991), no information is available on dietary lysine requirement of fingerling *C. catla.* Therefore, this study was undertaken to work out the dietary lysine requirement of the fingerling stage of this important cultivated fish species.

MATERIALS AND METHODS

Experimental diets

Six isonitrogenous (33% crude protein) and isocaloric (16.72 kJ/g gross energy) amino acid test diets using casein (fat-free), gelatin and crystalline L-amino acids with graded

*Published in '*Fish Physiology and Biochemistry*'39: 503-512 (2013)

levels of L-lysine (1.25, 1.50, 1.75, 2.00, 2.25 and 2.50% dry diet) were prepared (Table 1). Diets were designated as $L_{1.25}$, $L_{1.5}$, $L_{1.75}$, L_2, $L_{2.25}$ and $L_{2.5}$. The levels of lysine in the amino acid test diets were fixed on the basis of information available on other two Indian major carps (Ahmed and Khan 2004b; Abidi and Khan 2010a). Crystalline L-amino acids, excluding the test amino acid lysine, were used to simulate the amino acid profile of the experimental diets to that of 33% whole chicken egg protein. The lysine content contributed by the casein and gelatin was 1.07 and 0.17% of the dry diet, respectively. To make the intended concentrations of dietary lysine in the amino acid test diets, the amount of lysine was increased at the expense of glycine on protein basis. Analyzed amino acid composition (% dry diet) of the experimental diets is presented in Table 2. Method of preparation of experimental diets has been discussed under the General Methodology section (pages 9-10).

Experimental design and feeding trial

Source of the fish, their acclimation and details of the general experimental design has already been discussed under the General Methodology section (page 8).

Fingerling *C. catla* (3.65±0.05 cm; 0.58±0.02 g) were taken from the above acclimated fish lot and stocked in triplicate groups in 70 L circular polyvinyl troughs (water volume 55 L) fitted with a continuous water flow-through (1-1.5 L/min) system at the rate of 25 fish per trough for each dietary treatment level. Fish were fed test diets in the form of dry crumbles (500 µm) to apparent satiation thrice a day at 08.00, 12.30 and 17.30h. Initial and weekly weights were recorded on a top-loading balance (Precisa 120A; 0.1 mg sensitivity, Oerlikon AG, Zurich, Switzerland) after anaesthetizing the fish with tricaine methane sulfonate (MS-222; 100 µg/ml). Fish were deprived of feed on the day they were weighed. The feeding trial lasted for 12 weeks. Faecal matter was siphoned before every feeding.

Water quality parameters

Water quality parameters were recorded daily during the feeding trial (APHA 1992). The average water temperature, dissolved oxygen, free carbon dioxide, pH, total ammonia

nitrogen, nitrites and total alkalinity based on daily measurements were 26.8±1.6°C, 6.8±0.9 mg/L, 7.9±2.1 mg/L, 7.1±0.5, 0.29±0.03 mg/L, 0.07±0.004 mg/L and 68.1±2.41 mg/L.

Sample collection

At the end of the 12-week feeding trial, four fish from each replicate of the treatment (n=3x4) were anesthetized with MS-222 (Tricaine Methane Sulphonate; 100 µg/ml) before taking the body measurements. Liver and viscera of each specimen was carefully removed and weights of fish, viscera and liver were recorded to calculate viscerosomatic index (VSI), HSI and condition factor (CF).

Chemical analyses

Proximate composition of casein, gelatin, experimental diets, and initial and final carcass was estimated using standard methods (pages 10-11). Gross energy content was determined on a Gallenkamp Ballistic Bomb Calorimeter as per the method described on page 12. Amino acid analysis of casein, gelatin, experimental diets (Table 2), initial and final fish carcass was done using an automatic amino acid analyzer detailed earlier (page 12). At the beginning of the feeding trial, 60 fish were randomly sampled, killed and pooled together. Six subsamples of a pooled sample were analyzed for initial carcass composition. At the end of the experiment, 20 fishes from each replicate of dietary treatments were randomly collected, sacrificed with an overdose of MS-222 and pooled separately. Three subsamples of the pooled samples were analyzed for final carcass composition.

Determination of RNA and DNA

RNA and DNA were determined by the method of Schneider (1957) as detailed earlier on page 13.

Evaluation of growth parameters

Calculation of various growth parameters was made according to the standard definitions

as described under the General Methodology section (pages 13-14).

Statistical analyses

Statistical analyses of growth data were done using procedures as detailed earlier on page 14.

RESULTS

Growth performance

Data related to absolute weight gain (AWG), feed conversion ratio (FCR), protein deposition (PD) and lysine retention efficiency (LRE%) are illustrated in Table 3. The second-degree polynomial regression analysis of the above parameters at 95% maximum response exhibited their best values at 1.8 (Fig. 1), 1.9, 1.7 (Fig. 2) and 1.7% lysine of the dry diet. The equations employed to establish the second-degree polynomial relationship of each variable is as under:

$$Y = -10.63X^2 + 43.11X-32.65 \ (R^2=0.994), \ 1.8\% \ (AWG);$$

$$Y=3.07X^2-12.52X+14.13 \ (R^2=0.972), \ 1.9\% \ (FCR);$$

$$Y=-0.37X^2+1.44X-1.11 \ (R^2=0.935), \ 1.7\% \ (PD);$$

$$Y=-71.41 \ X^2+279.65X-201.92 \ (R^2= 0.918), \ 1.7\% \ (LRE)$$

The amino acid test diets were well accepted by the fishes of all the treatment groups and feed intake was not significantly affected by the dietary lysine concentrations (Table 3).

Carcass composition

Carcass composition of fish fed varying levels of dietary lysine is presented in Table 4. These parameters were affected by the varying concentrations of dietary lysine. Carcass fat content decreased linearly with the increase in lysine up to 2.0% of the dry diet (L_2), beyond this remained almost unchanged. Moisture content exhibited the reverse trend in

contrast to carcass fat. Carcass protein attained the highest value (15.6%) for the group fed dietary lysine at 1.75% ($L_{1.75}$). Dietary treatments did not influence carcass ash content. No mortality was recorded during the entire length of the feeding trial.

Nucleic acid indices

Nucleic acid indices were affected by the varying levels of dietary lysine (Table 4). Muscle DNA concentration was found to decrease with the increase in dietary lysine levels up to 2.0% (L_2) and a slight increase was noted for the groups fed higher levels of lysine at 2.25 and 2.5% dry diet ($L_{2.25}$-$L_{2.5}$). The muscle RNA concentration was found to increase with the increased inclusion of lysine up to 1.75% of the dry diet ($L_{1.75}$) and decreased in groups fed higher dietary lysine concentrations at 2.0, 2.25 and 2.5% (L_2, $L_{2.25}$ and $L_{2.5}$). The data for RNA/DNA ratio were also subjected to second-degree polynomial regression analysis which at 95% of maximum response exhibited the lysine requirement at 1.7% of the dry diet. The second-degree polynomial regression equation employed to calculate the requirement was $Y=-5.31X^2+20.74X-15.87$ ($R^2=0.870$).

Somatic indices

Somatic indices including HSI, VSI and CF are depicted in Table 4. Fish fed lowest level of dietary lysine ($L_{1.25}$) had highest HSI value (0.98). Viscerosomatic index was found to decrease with the increase of dietary lysine up to 2.0% (L_2) and then increased in fish fed at higher levels (2.25 and 2.5%) of dietary lysine ($L_{2.25}$-$L_{2.5}$). Graded levels of dietary lysine had an impact on condition factor. Lowest value of CF (0.93) was recorded for the group fed 1.25% dietary lysine ($L_{1.25}$). However, it improved (0.93-1.58) with the increase in dietary lysine concentrations up to 2.0% (L_2) and declined for the groups receiving higher dietary lysine concentrations ($L_{2.25}$-$L_{2.5}$).

Amino acid composition of fish carcass

The amino acid composition of fish carcass fed diets with increasing levels of lysine is given in Table 5. Lysine content of fish carcass was significantly affected by dietary lysine levels. Fish fed diet containing 1.75% lysine ($L_{1.75}$) showed highest lysine content.

Whereas lowest lysine content was recorded in group receiving 1.25% dietary lysine ($L_{1.25}$). However, no significant ($P>0.05$) change in concentrations of other amino acids was noted.

Second-degree polynomial regression analysis at 95% maximum and minimum response of AWG, FCR, PD, LRE and RNA/DNA ratio against dietary lysine concentrations yielded the optimal values between 1.7-1.9% of the dry diet, corresponding to 5.2-5.8% of dietary protein.

DISCUSSION

Since absolute weight gain and protein deposition are the key parameters for estimating requirement (Encarnacao et al. 2004), these parameters were used to estimate the lysine requirement of fingerling *C. catla*. The second-degree polynomial regression analysis of AWG and PD exhibited the lysine requirement of fingerling *C. catla* in the range of 1.7-1.8% dry diet. The requirement obtained in this study is lower than that reported for common carp, *C. carpio* 2.2%; rohu, *L. rohita* 2.3%; rainbow trout, *Oncorhynchus mykiss* 2.4%; Atlantic Salmon, *Salmo salar* 2.4%; pacific salmon, *Oreochromis spp.* 2.2% (NRC 2011) and comparable to the requirement of tilapia *O. spp.* 1.6%; channel catfish, *Ictalurus punctatus* 1.6% of the dry diet (NRC 2011). The above discrepancies in lysine requirements among fishes may be attributed to differences in metabolic requirements of the species and daily protein consumption by fish, dietary formulations, and feeding regimes used in the classical dose-response experiments (Cowey 1993; Fagbenro et al. 1998). In addition, use of different mathematical methods for fitting the response and estimating requirement results in different estimates of amino acid requirements (Rodehutscord and Pack 1999).

The lysine requirement of fingerling *C. catla* worked out during this study (1.7-1.8% dry diet) is lower than the requirement reported by Ravi and Devaraj (1991) for fry stage of this fish (2.5% dry diet). Higher amino acid requirement estimates are associated with the smaller size fish whereas it becomes comparatively lower with the advancing stages probably as a result of the higher rates of protein synthesis and growth displayed

by smaller individuals (Houlihan et al. 1986; Moltschaniwskyj and Carter 2010). This may be the reason for the differences in the lysine requirement as fish under study was in fingerling stage requiring lower dietary nutrient requirements for the metabolical and physiological activities than the fry stage of the fish in the study conducted by Ravi and Devaraj (1991). Differences in the lysine requirement of similar species may be due to differences in experimental design, available dietary energy and the composition of the specific dietary protein (Wilson 1984). Ravi and Devaraj (1991) have used the crystalline L-amino acids in unbound form hampering the amino acid utilization by lowering its gut retentivity, hence yielding to a higher requirement estimates. However, in present study, the crystalline L-amino acids were coated with casein and gelatin that have promoted the retention time of the amino acid in the gut leading to more efficient utilization of the ingested amino acids. These methodological biases may probably be the reasons for the differences obtained in the requirement estimates of the two studies.

In the present study, absolute weight gain of fingerling *C. catla* was found to improve quadratically up to 1.8% dietary lysine ($Y_{95\%max}$) and then declined with further increase in dietary lysine levels. This trend of weight gain is in agreement with the results reported by earlier workers (Ahmed and Khan 2004b; Wang et al. 2005; Mai et al. 2006a; Dairiki et al. 2007; Bicudo et al. 2009; Abidi and Khan 2010a; Xie et al. 2012, Furuya et al. 2012). The reduction in growth of fish fed higher than required levels of dietary lysine may either be due to the stress caused by excess amount of amino acid in the body of the fish leading to extra energy expenditure towards deamination and excretion (Abidi and Khan 2010a).

The RNA/DNA ratio has been considered to be a promising indicator of growth as it is closely related to the rate of protein synthesis (Mustafa 1977; 1979; 1983; Mustafa and Jafri 1977; Mustafa and Mittal 1982; Mustafa and Zofair 1985; Bulow 1987; Mustafa et al. 1991; Buckley et al. 1999; Tanaka et al. 2007; Abidi and Khan 2009). In the present study, muscle RNA/DNA ratio was found to improve quadratically up to 1.7% lysine of the dry diet. The dietary imbalance of amino acids affects the efficiency of protein utilization. Lysine is primarily used for protein deposition (Firman 2004) which in this study was found to improve quadratically with the increased inclusion of dietary lysine

up to 1.7% ($Y_{95\%max}$) and a reduction was noted for the groups fed dietary lysine above this level. Carcass protein also followed the same trend. Carcass lipid responded negatively with the increasing concentrations of dietary lysine up to 2.0% (L_2). This indicates that a better dietary amino acid balance probably prevents the selective catabolism of amino acids and consequently increases protein synthesis, while decreasing the accumulation of lipid reserves (Tantikitti and Chimsung 2001; Conceicao et al. 2003). The declining trend of carcass fat content as observed in this study may also be due to the fact that lysine is the precursor of L-carnitine (Rebouche 1992) which acts as a lipolytic factor thus contributing to a reduction of body fat deposition (Dias et al. 2001). It also facilitates removal of short-chain organic acids from mitochondria, thereby, freeing intramitochondrial coenzyme A to participate in the β-oxidation and tricarboxylic acid cycle pathways which could avoid accumulation of lipid in fish body (Ozorio et al. 2003). The low carcass lipid content recorded for fish fed incremental levels of dietary lysine as evident in this study, is in agreement with Ruchimat et al. (1997); Keshavanth and Renuka (1998); Mai et al. (2006a) and Ma et al. (2007). Common adverse effects of lysine deficiency in fish are slow growth rate and poor protein utilization. These deficiency signs were also recorded in this study. Hence, optimizing dietary lysine is prerequisite for growth and protein deposition in fingering *C. catla*.

Second-degree polynomial regression analysis of AWG and PD data exhibited the lysine requirement between 1.7-1.8% dry diet which is recommended to prepare lysine-balanced, cost-effective practical feeds for intensive aquaculture of this valuable Indian major carp species.

SUMMARY

A twelve-week experiment was conducted to quantify dietary lysine requirement of fingerling *Catla catla* (3.65±0.05 cm; 0.58±0.02 g) by feeding casein-gelatin based diets (33% crude protein; 16.72 kJ/g gross energy) with six levels of L-lysine (1.25, 1.50, 1.75, 2.00, 2.25 and 2.50% dry diet). The experiment was conducted in eighteen 70 L indoor polyvinyl circular troughs provided with a water flow-through system (1-1.5 L/min). Absolute weight gain (AWG), feed conversion ratio (FCR), protein deposition (PD),

lysine retention efficiency (LRE%) and RNA/DNA ratio were used as the response criteria. Second-degree polynomial regression analysis at 95% maximum and minimum response of AWG and FCR data exhibited the lysine requirement between 1.8-1.9% dry diet, corresponding to 5.5-5.7% dietary protein. Regression analysis of PD, LRE and RNA/DNA ratio yielded the requirement between 1.7-1.8% dry diet, corresponding to 5.2-5.5% dietary protein. Since absolute weight gain and protein deposition are the key parameters for estimating nutrient requirement, these tools were used to recommend the lysine requirement of fingerling *C. catla* which ranges between 1.7-1.8% dry diet. Data generated during this study will be useful to formulate lysine balanced feed for intensive culture of this fish.

Table 1 Composition of the experimental diets

	Dietary lysine levels (%)					
Ingredients (g/100 g)	**1.25 ($L_{1.25}$)**	**1.50 ($L_{1.5}$)**	**1.75 ($L_{1.75}$)**	**2.00 (L_2)**	**2.25 ($L_{2.25}$)**	**2.50 ($L_{2.5}$)**
Casein[a] (fat-free)	13	13	13	13	13	13
Gelatin[b]	4.33	4.33	4.33	4.33	4.33	4.33
Dextrin	33.61	33.62	33.63	33.65	33.66	33.67
Amino acid mixture[c]	19.90	19.89	19.88	19.87	19.87	19.89
Corn oil	5	5	5	5	5	5
Cod liver oil	2	2	2	2	2	2
Mineral mix[d,f]	4	4	4	4	4	4
Vitamin mix[e,f]	3	3	3	3	3	3
α-Cellulose	5.16	5.16	5.16	5.15	5.15	5.14
Carboxymethyl cellulose	10	10	10	10	10	10
Total	100	100	100	100	100	100
Analyzed crude protein	33.51	33.46	33.17	33.38	32.62	32.74
Digestible energy[g] (kJ/g, dry diet)	14.33	14.33	14.33	14.33	14.33	14.33
Calculated gross energy (kJ/g, dry diet)	16.72	16.72	16.72	16.72	16.72	16.72

[a]Crude Protein (76%); [b]Crude Protein (96%); [c]Amino acid mixture (g/100 g dry diet) arginine 1.289, histidine 0.317, isoleucine 1.867, leucine 1.653, lysine variable, methionine 0.897, cystine 0.738, phenylalanine 0.353, tyrosine 0.819, threonine 0.895, tryptophan 0.396, valine 1.459, alanine 1.069, aspartic acid 0.079, proline 0.827, glycine variable; (Loba Chemie, India); [f]Halver (2002); [d]Mineral mixture (g/100 g of mineral mixture) calcium biphosphate 13.57; calcium lactate 32.69; ferric citrate 02.97; magnesium sulphate 13.20; potassium phosphate (dibasic) 23.98; sodium biphosphate 08.72; sodium chloride 04.35; aluminium chloride. $6H_2O$ 0.0154; potassium iodide 0.015; cuprous chloride 0.010; manganous sulphate. H_2O 0.080; cobalt chloride. $6H_2O$ 0.100; zinc sulphate. $7H_2O$ 0.40; [e]Vitamin mixture (g/100 g dry diet) choline chloride 0.500; inositol 0.200; ascorbic acid 0.100; niacin 0.075; calcium pantothenate 0.05; riboflavin 0.02; menadione 0.004; pyridoxine hydrochloride 0.005; thiamin hydrochloride 0.005; folic acid 0.0015; biotin 0.0005; alpha-tocopherol 0.04; vitamin B_{12} 0.00001; 2 g α-cellulose; [g]Digestible energy was calculated on the basis of physiological fuel values 18.83, 14.64 and 35.56 kJ/g for protein, carbohydrate and fat, respectively (Jauncey 1982).

Table 2 Analyzed amino acid composition of the reference protein and experimental diets (%)[a]

Amino acids	33% whole chicken egg protein	1.25 ($L_{1.25}$)	1.50 ($L_{1.5}$)	1.75 ($L_{1.75}$)	2.00 (L_2)	2.25 ($L_{2.25}$)	2.50 ($L_{2.5}$)
EAAs							
Arginine	2.11	2.10	2.16	2.02	2.12	2.15	2.13
Histidine	0.69	0.70	0.71	0.69	0.70	0.72	0.69
Isoleucine	2.64	2.66	2.65	2.65	2.66	2.68	2.67
Leucine	3.04	3.06	3.11	3.10	3.12	3.11	3.12
Lysine	2.38	1.22	1.46	1.73	1.96	2.22	2.47
Methionine	1.36	1.37	1.35	1.38	1.37	1.36	1.37
Phenylalanine	2.08	2.12	2.13	2.11	2.14	2.10	2.11
Threonine	1.43	1.47	1.45	1.45	1.44	1.46	1.47
Tryptophan	0.50	0.53	0.52	0.53	0.53	0.51	0.52
Valine	2.41	2.43	2.44	2.46	2.41	2.42	2.44
NEAAs							
Cystine	0.79	0.81	0.83	0.80	0.82	0.81	0.83
Tyrosine	1.49	1.51	1.52	1.53	1.50	1.51	1.52
Alanine	1.90	1.89	1.91	1.88	1.92	1.87	1.88
Aspartic acid	1.14	1.16	1.15	1.14	1.13	1.16	1.17
Glycine	4.27	7.54	7.28	7.02	6.76	6.51	6.25
Proline	2.77	2.81	2.83	2.80	2.78	2.81	2.74

[a]Determined by Hitachi L-8800 Automatic Amino Acid Analyzer

Table 3 Growth performance, feed conversion, protein deposition and lysine retention efficiency of fingerling *C. catla* fed diets with varying levels of lysine[a,b]

	Dietary lysine levels (%)						
	1.25 ($L_{1.25}$)	1.50 ($L_{1.5}$)	1.75 ($L_{1.75}$)	2.00 (L_2)	2.25 ($L_{2.25}$)	2.50 ($L_{2.5}$)	Quadratic Pr>F[c]
Average initial weight (g)	0.58±0.07	0.61±0.02	0.59±0.01	0.59±0.008	0.60±0.009	0.59±0.01	0.779[b]
Average final weight (g)	3.30±0.02	5.46±0.06	6.73±0.05	7.27±0.07	6.76±0.11	5.77±0.04	0.00008
Absolute weight gain (g/fish)	4.69±0.05	7.95±0.04	10.41±0.09	11.32±0.06	10.27±0.07	8.78±0.03	0.0005
Feed intake (g/fish)	9.28±0.12	9.74±0.11	9.33±0.14	9.89±0.16	9.91±0.15	9.62±0.12	0.432[b]
Feed conversion ratio	3.41±0.11	2.01±0.04	1.52±0.02	1.48±0.02	1.61±0.04	1.86±0.03	0.011
Protein deposition	0.11±0.02	0.23±0.01	0.32±0.02	0.30±0.02	0.24±0.02	0.20±0.01	0.018
Lysine retention efficiency%	34.71±0.51	58.84±0.58	76.15±0.52	67.15±0.51	61.21±0.52	54.93±0.47	0.041

[a]Mean values of 3 replicates ± SEM. [b]Not statistically significant (P>0.05). [c]Significance probability associated with the F statistic.

Table 4 Carcass composition (wet basis), muscle RNA/DNA ratio and somatic indices of fingerling *C. catla* fed diets with varying levels of lysine[a,b]

	Dietary lysine levels (%)							
	Initial	1.25 ($L_{1.25}$)	1.50 ($L_{1.5}$)	1.75 ($L_{1.75}$)	2.00 (L_2)	2.25 ($L_{2.25}$)	2.50 ($L_{2.5}$)	Quadratic Pr>F[c]
Moisture (%)	79.2±0.3	76.3±0.6	77.1±0.7	77.6±0.7	78.2±0.6	78.1±0.7	78.0±0.7	0.001
Protein (%)	12.4±0.06	12.5±0.05	14.7±0.07	15.6±0.05	14.3±0.06	14.0±0.09	13.5±0.08	0.147
Fat (%)	3.8±0.11	4.9±0.02	4.3±0.06	3.3±0.08	2.7±0.04	2.6±0.05	2.6±0.03	0.002
Ash (%)	2.3±0.08	2.3±0.03	2.4±0.02	2.3±0.04	2.4±0.03	2.4±0.02	2.3±0.02	0.591[b]
RNA (µg/100 mg dry weight basis)	849±5	724±4	978±6	1197±4	1084±5	916±3	827±4	0.048
DNA (µg/100 mg dry weight basis)	431±4	421±3	314±3	249±2	257±2	261±3	266±2	0.013
RNA/DNA ratio	1.9±0.06	1.7±0.02	3.1±0.07	4.8±0.04	4.2±0.05	3.5±0.06	3.1±0.02	0.046
Viscerosomatic index%	5.9±0.04	6.5±0.02	5.6±0.02	4.7±0.05	4.4±0.03	4.7±0.02	5.0±0.02	0.002
Hepatosomatic index%	0.8±0.02	1.0±0.03	0.7±0.02	0.7±0.02	0.6±0.04	0.5±0.03	0.4±0.02	0.018
Condition factor	1.0±0.03	0.9±0.02	1.2±0.05	1.4±0.06	1.6±0.03	1.5±0.02	1.4±0.08	0.002

[a]Mean values of 3 replicates ± SEM. [b]Not statistically significant (P>0.05). [c]Significance probability associated with the F statistic.

Table 5 Analyzed carcass amino acid composition (g/100 g dry matter) of fingerling *C. catla* fed diets with varying levels of lysine[a,b]

Amino acids	1.25 ($L_{1.25}$)	1.50 ($L_{1.5}$)	1.75 ($L_{1.75}$)	2.00 (L_2)	2.25 ($L_{2.25}$)	2.50 ($L_{2.5}$)	Quadratic Pr>F[d]
EAAs							
Arginine	4.91 ±0.05	4.92 ±0.09	5.11 ±0.11	5.03 ±0.14	5.01 ±0.12	4.99 ±0.14	0.249[c]
Histidine	2.10 ±0.03	2.11 ±0.05	2.10 ±0.07	2.09 ±0.06	2.06 ±0.04	2.08 ±0.02	0.504[c]
Isoleucine	2.21 ±0.06	2.20 ±0.02	2.22 ±0.09	2.20±0.04	2.21 ±0.03	2.19 ±0.04	0.067[c]
Leucine	4.12 ±0.04	4.14 ±0.07	4.15 ±0.16	4.14 ±0.14	4.15 ±0.12	4.13 ±0.15	0.053[c]
Lysine	4.25 ±0.11	4.87 ±0.09	5.64±0.13	5.53 ±0.11	5.47 ±0.14	5.32 ±0.12	0.013
Methionine+cystine	1.68 ±0.04	1. 67 ±0.07	1.66 ±0.11	1.68 ±0.13	1.67 ±0.09	1.66 ±0.05	0.196[c]
Phenylalanine+tyrosine	4.79 ±0.17	4.80 ±0.15	4.78 ±0.14	4.81 ±0.18	4.79 ±0.13	4.76 ±0.11	0.329[c]
Threonine	2.48 ±0.04	2.50 ±0.06	2.46 ±0.08	2.47 ±0.11	2.46 ±0.13	2.49 ±0.08	0.787[c]
Tryptophan	0.53 ±0.02	0.51 ±0.01	0.54±0.02	0.52±0.03	0.53 ±0.02	0.54±0.02	0.819[c]
Valine	2.69 ±0.02	2.71 ±0.05	2.68 ±0.03	2.69 ±0.11	2.72 ±0.06	2.70 ±0.08	0.461[c]
NEAAs							
Alanine	3.87 ±0.13	3.91 ±0.11	3.93 ±0.09	3.88 ±0.16	3.85 ±0.14	3.83 ±0.10	0.063[c]
Aspartic acid	5.57 ±0.06	5.61 ±0.17	5.64 ±0.12	5.62 ±0.14	5.59 ±0.11	5.57 ±0.15	0.075[c]
Glycine	4.49 ±0.10	4.51 ±0.08	4.59 ±0.14	4.54 ±0.10	4.52 ±0.08	4.49 ±0.12	0.006[c]
Proline	2.86 ±0.04	2.85 ±0.09	2.87 ±0.07	2.84 ±0.03	2.86 ±0.06	2.85 ±0.09	0.378[c]
Serine	2.30 ±0.02	2.31 ±0.04	2.28 ±0.05	2.26 ±0.08	2.27 ±0.04	2.30 ±0.11	0.104[c]

[a]Determined by Hitachi L-8800 Automatic Amino Acid Analyzer. [b]Mean values of 3 replicates ± SEM. [c]Not statistically significant (P>0.05). [d]Significance probability associated with the F statistic.

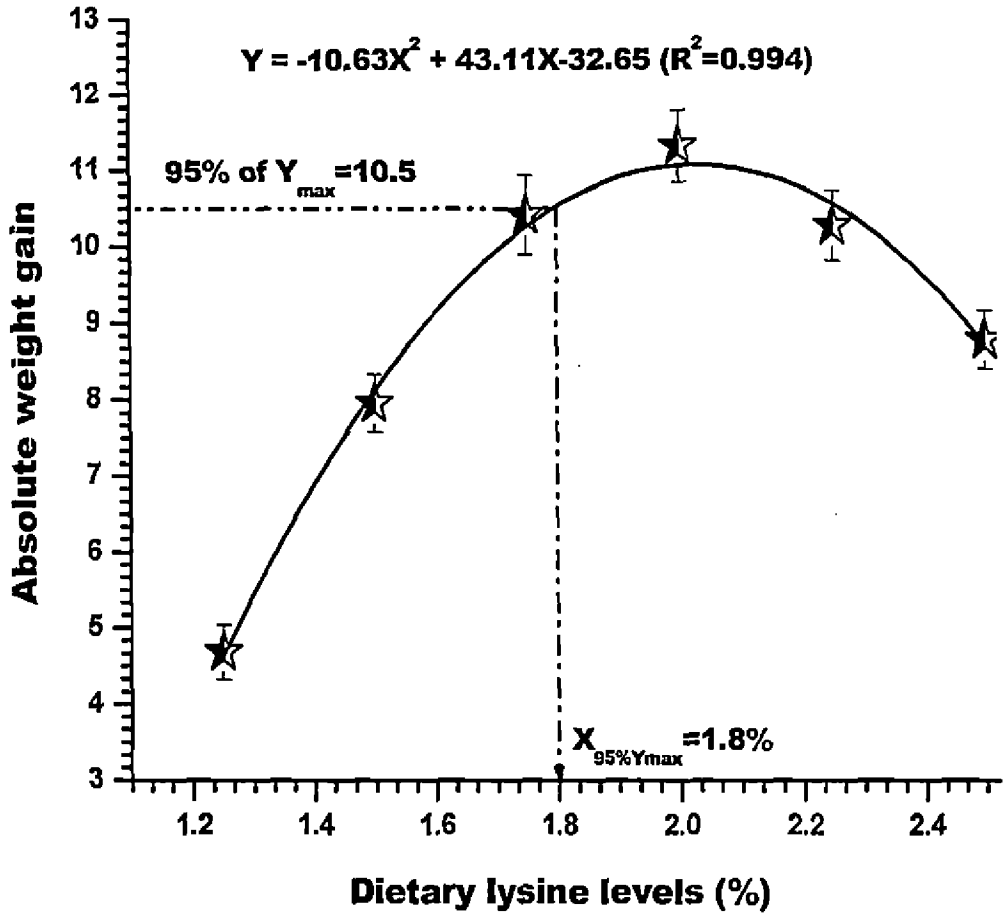


Fig. 1 Second-degree polynomial relationship of dietary lysine to absolute weight gain

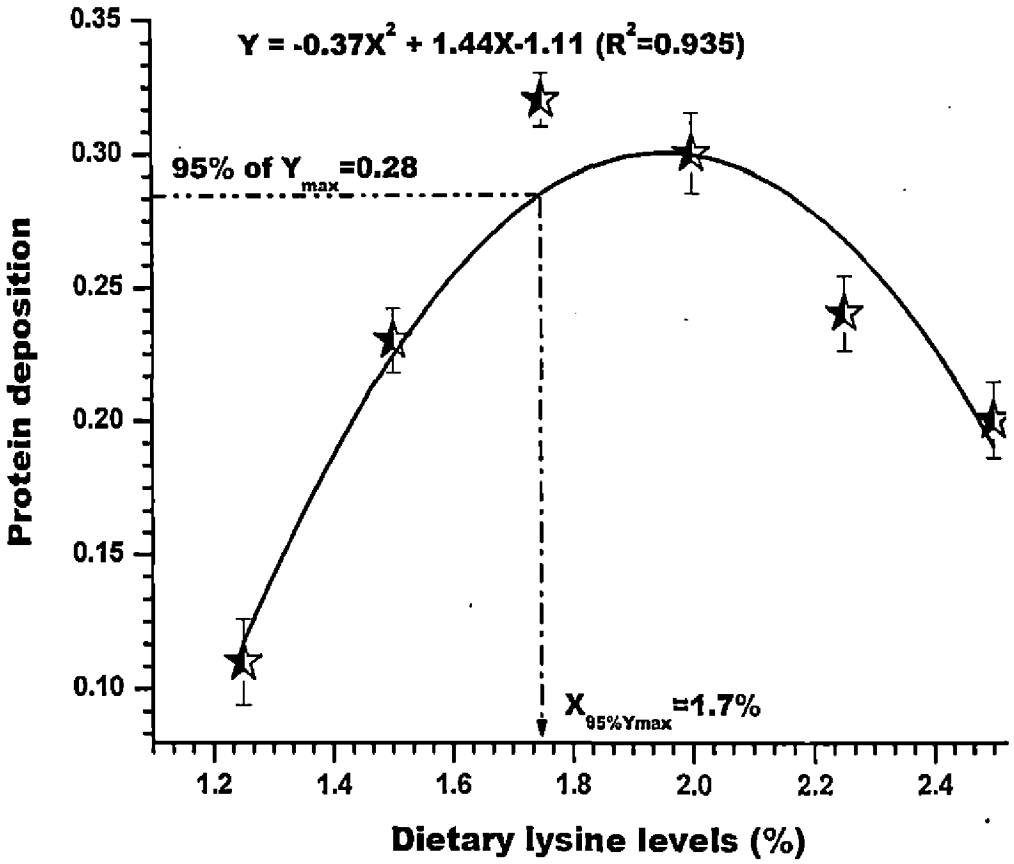


Fig. 2 Second-degree polynomial relationship of dietary lysine to protein deposition

CHAPTER 3

CHAPTER 3

DIETARY ISOLEUCINE REQUIREMENT OF FINGERLING CATLA, *CATLA CATLA* (HAMILTON) BASED ON GROWTH, PROTEIN PRODUCTIVE VALUE, ISOLEUCINE RETENTION EFFICIENCY AND CARCASS COMPOSITION*

INTRODUCTION

Successful and sustainable aquaculture depends upon the provision of nutritionally-balanced, environmental friendly and economically viable practical feeds (Singh et al. 2006) which are the crucial elements in the culture of aquatic animals. Therefore, knowledge on nutrition and practical feeding of fish is essential for successful aquaculture. In aquaculture, feed is often the single largest operating cost item and can represent over 50% of the operating costs in intensive aquaculture (El-Sayed 2004). Protein is a basic and most expensive component of fish feeds, both in terms of quantity and quality. Fish require not only a minimum level of protein but also that the essential amino acids are balanced to meet the requirements of each single one (Dacosta-Calheiros et al. 2003). The adjustment of essential amino acid as per the dietary needs of fish not only improves nutritional efficiency and feed-conversion efficiency but also influence the utilization of other nutrients. If the essential amino acid requirements of fish species are known, it would be possible to meet these needs by using combinations of different cost-effective protein ingredients. The complete 10 essential amino acid requirements have been established for only a limited number of cultured fish species such as mrigal, *Cirrhinus mrigala*; Atlantic Salmon, *Salmo salar*; Common carp, *Cyprinus carpio*; rohu, *Labeo rohita*; tilapia, *Oreochromis spp.*; channel catfish, *Ictalurus punctatus;* rainbow trout, *Oncorhynchus mykiss* and pacific salmon, *Oncorhynchus spp.* (NRC 2011).

Of the ten essential amino acids, evaluation of isoleucine requirement is of particular importance because isoleucine along with the other two branched-chain amino acids acts as nutrient regulator of protein synthesis and protein degradation. It is also involved in the insulin biosynthesis and secretion (Kimball and Jefferson 2006). In

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*Published in '*Aquaculture International*' 21, 1243-1259 (2013)

addition to this, it helps in energy production in the body and has been found to reduce twitching and tremors in animals (Braverman et al. 2003). It is the first limiting of the branched chain amino acids in meat and bone meal as well as the first limiting of those amino acids not available in commercial feed-grade form (Wang et al. 1997).

The dietary requirements of isoleucine have been estimated for different fish species including chinook salmon, *O. tshawytscha* (Chance et al. 1964); channel catfish, *I. punctatus* (Wilson et al. 1980); rainbow trout, *O. mykiss* (Ogino 1980); Mossambique tilapia, *O. mossambicus* (Jauncey et al. 1983); rohu, *L. rohita* (Murthy and Varghese 1996a; Khan and Abidi 2007a); white sturgeon, *Acipenser transmontanus* (Ng and Hung 1995); red sea bream, *Chrysophrys major* (Forster and Ogata 1998); European sea bass, *Dicentrarchus labrax*; gilthead seabream, *Sparus aurata* and turbot, *Scophthalmus maximus* (Kaushik 1998); Atlantic salmon, *Salmo salar* (Rollin 1999), mrigal, *C. mrigala* (Benakappa and Varghese 2003; Ahmed and Khan 2006) and grass carp, *Ctenopharyngodon idella* (Di et al. 2009).

Although information on isoleucine requirement of fry *C. catla* exists (Ravi and Devaraj 1991), no information on isoleucine requirement of fingerling stage of *C. catla* is available. Hence, this study was undertaken to determine the isoleucine requirement of fingerling *C. catla* using the dose-response method.

In the present study, absolute weight gain, feed conversion ratio, protein productive value, isoleucine retention efficiency and carcass protein were used as growth indicators to estimate the isoleucine requirement of this fish. Relevance of RNA and DNA data in growth and condition assessment of fish has been documented by a number of authors (Mustafa 1977; Bulow 1987; Abidi and Khan 2009). Considering the significance of RNA/DNA ratio as an index of protein synthetic machinery in cells and therefore as a sensitive indicator of fish growth in response to nutritional status (Clemmesen 1994; Mustafa and Jafri 1977; Mustafa and Mittal 1982; Mustafa et al. 1991), this parameter was also employed, in addition to growth parameters, to quantify the dietary isoleucine requirement.

MATERIALS AND METHODS

Experimental diets

Six isonitrogenous (33% crude protein) and isocaloric (16.72 kJ/g gross energy) amino acid test diets using casein (fat-free), gelatin and crystalline L-amino acids with graded levels of isoleucine (0.5, 0.75, 1.0, 1.25, 1.5 and 1.75% of dry diet) were prepared (Table 1; Table 2). Diets were named as $I_{0.5}$, $I_{0.75}$, I_1, $I_{1.25}$, $I_{1.5}$ and $I_{1.75}$. The levels of isoleucine in the amino acid test diets were fixed on the basis of information available on other two Indian major carps (Murthy and Varghese 1996a; Khan and Abidi 2007a; Benakappa and Varghese 2003; Ahmed and Khan 2006). The amino acid composition of the experimental diets simulated to that of 33% whole chicken egg protein excluding the test amino acid isoleucine. Analyzed amino acid composition of 33% whole chicken egg protein reflected the arginine, histidine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, cystine, tyrosine, alanine, aspartic acid, glutamic acid, proline and serine contents to be 2.11, 0.69, 3.04, 2.38, 1.36, 2.08, 1.43, 0.5, 2.41, 0.79, 1.49, 1.9, 1.14, 2.34, 2.77 and 0.57% of the dry matter, respectively. The isoleucine content contributed by the casein and gelatin in experimental diets was 0.45 and 0.03% dry diet, respectively. The amount of isoleucine was increased at the expense of glycine, on nitrogen to nitrogen to attain the intended concentrations of dietary isoleucine in the amino acid test diets. Method of preparation of experimental diets has been discussed under the General Methodology section (pages 9-10).

Experimental design and feeding trial

Source of the fish, their acclimation and details of the general experimental design has already been discussed under the General Methodology section (page 8).

Fingerling *C. catla* (4.25±0.15 cm, 0.61±0.04 g) were taken from the above acclimated fish lot and stocked in triplicate groups at the rate of 25 fish per trough for each dietary treatment level in 70 L circular polyvinyl troughs (water volume 55 L) fitted with a continuous water flow-through (1-1.5 L/min) system. Fish were fed the test diets in the form of dry crumbles (500 µm) to apparent satiation thrice daily at 08:00, 12:30

and 17:30h. Initial and weekly weights were recorded on a top-loading balance (Precisa 120A; 0.1 mg sensitivity, Oerlikon AG, Zurich, Switzerland). Fish were deprived of feed on the day they were weighed. The feeding trial lasted for 12 weeks. Faecal matter was siphoned before every feeding. Water quality indices were recorded following standard methods (APHA 1992). The range of water temperature, dissolved oxygen, free carbon dioxide, pH, total ammonia nitrogen, nitrites and total alkalinity, based on daily measurements, was 27.1-28.4°C, 6.4-7.1 mg/L, 5.8-10.1 mg/L, 7.2-7.6, 0.27-0.34 mg/L, 0.03-0.07 mg/L and 68.4-81.6 mg/L, respectively.

Sample collection

At the beginning of the feeding trial, 60 fish were randomly sampled, killed and pooled. Six subsamples of the pooled sample were analyzed for initial carcass composition. At the end of the experiment, 15 fishes from each replicate of all the dietary isoleucine treatments were randomly collected, sacrificed and pooled. Three subsamples of the pooled samples were analyzed for final carcass composition. Another five fish from each replicate (n=3x5) were anesthetized with MS-222 (Tricaine Methane Sulphonate; 100 µg/ml) and liver and viscera of each specimen were carefully removed. Weight of fish, viscera and liver were recorded to calculate viscerosomatic index (VSI), hepatosomatic index (HSI) and condition factor (CF). After taking the weight of fish, viscera and liver, white muscle tissue was removed from these fish. Three subsamples of each tissue sample were taken for the determination of RNA and DNA.

Chemical analyses

Proximate composition of casein, gelatin, experimental diets, and initial and final carcass was estimated using standard methods as detailed on pages 10-11. Gross energy content was determined on a Gallenkamp Ballistic Bomb Calorimeter as per the method described on page 12. Amino acid analysis of casein, gelatin, experimental diets (Table 2), initial and final fish carcass was done using an automatic amino acid analyzer as given under the General Methodology section (page 12).

Determination of RNA and DNA

RNA and DNA were determined by the method of Schneider (1957) as detailed earlier on page 13.

Evaluation of growth parameters

Calculation of various growth parameters was made according to the standard definitions as described under the General Methodology section (pages 13-14).

Statistical analyses

Statistical analyses of growth data were done using procedures as detailed earlier (page 14).

RESULTS

Absolute weight gain (AWG), feed conversion ratio (FCR), protein productive value (PPV), isoleucine retention efficiency (IRE) and RNA/DNA ratio of fingerling *C. catla* fed diets with graded levels of isoleucine over the 12-week feeding trial were found to increase significantly with the increase in dietary isoleucine concentrations up to 1.25% (Table 3). The best values of AWG (6.52 g/fish), FCR (1.45), PPV (0.35), IRE (74.13%) and RNA/DNA ratio (5.20) were observed in fish fed diet $I_{1.25}$. No significant differences ($P>0.05$) in growth parameters were recorded in fish fed 1.5% isoleucine diet. However, further increase in dietary isoleucine concentration (1.75%) led to significant fall ($P<0.05$) in growth parameters. Feed intake did not differ significantly among treatment groups. No mortality was recorded in all the treatment groups.

Dietary isoleucine concentrations had an impact on carcass composition of fingerling *C. catla* (Table 4). Moisture content showed a reverse trend to that of the dietary isoleucine concentrations up to 1.25% ($I_{1.25}$). Carcass protein content was found to improve quadratically ($P<0.05$) with the increase in dietary isoleucine concentrations up to 1.25% with the highest value (16.54%) at this level ($I_{1.25}$). A further inclusion of dietary isoleucine ($I_{1.5}$-$I_{1.75}$) resulted a slight reduction ($P>0.05$) in carcass protein.

Carcass fat showed a decreasing trend up to 1.25% dietary isoleucine ($I_{1.25}$) beyond that no significant change was recorded. Carcass ash content remained almost the same except those fed diet $I_{0.5}$ and $I_{0.75}$.

Somatic indices including HSI, VSI and CF are illustrated in Table 4. The HSI did not show marked variations with the dietary isoleucine levels except for the fish receiving diet $I_{0.5}$. Fish fed the lowest level of dietary isoleucine ($I_{0.5}$) had the highest value of HSI. A negative correlation in VSI with the increasing concentrations of dietary isoleucine was noted up to 1.25% ($I_{1.25}$). It was found to increase with the further increase of dietary isoleucine ($I_{1.5}$-$I_{1.75}$). The CF improved with the increase in dietary isoleucine and was found to be highest at 1.25%. Fish fed higher levels of dietary isoleucine at 1.5 ($I_{1.5}$) and 1.75% ($I_{1.75}$) showed significant decline in CF.

In order to find precise isoleucine requirement, AWG, PPV, IRE, RNA/DNA ratio and carcass protein data were subjected to quadratic regression analysis which at 95% maximum response exhibited the optimum dietary isoleucine requirement to range between 1.13-1.18% dry diets which is equivalent to 3.42-3.58% dietary protein. The quadratic equations employed for AWG (Fig. 1), PPV (Fig. 2), IRE (Fig. 3), RNA/DNA ratio (Fig. 4) and carcass protein (Fig. 5) are depicted in the respective figures.

DISCUSSION

Dietary isoleucine requirement of fingerling *C. catla* (3.42-3.58% dietary protein) worked out in this experiment is found to be higher than that reported for chinook salmon, *O. tshawytscha* 2.2% (Chance et al. 1964); common carp, *C. carpio* 2.5% (Nose 1979); channel catfish, *I. punctatus* 2.6% (Wilson et al. 1980); rainbow trout, *O. mykiss* 2.4% (Ogino 1980); Nile tilapia, *O. niloticus* 3.1% (Santiago and Lovell 1988); chum salmon, *O. keta* 2.4% (Akiyama and Arai 1993); coho salmon, *O. kisutch* 1.2% (Arai and Ogata 1993); white sturgeon, *A. transmontanus* 3.0% (Ng and Hung 1995); Japanese flounder, *P. olivaceus* 2.0% and red sea bream, *C. major* 2.2% (Forster and Ogata 1998); turbot, *S. maximus* 2.60% (Kaushik 1998); Atlantic salmon, *S. salar* 3.2% (Rollin 1999); mrigal, *C. mrigala* 3.12-3.15% (Benakappa and Varghese 2003, Ahmed and Khan 2006);

but lower than the requirement reported for milkfish, *Chanos chanos* 4.0% (Borlongan and Coloso 1993); grass carp, *C. idella* 4.0-4.23% (Di et al. 2009) and comparable to the requirement of rohu, *L. rohita* 3.75% (Khan and Abidi 2007a) and tilapia 3.45% (NRC 2011) of dietary protein. The above discrepancies in amino acid requirements of fish may be affected by fish size and age, adequate levels of other nutrients, flow rate, stock density, and the environmental and culture conditions adopted by different laboratories (Cowey and Luquet 1983; Kim et al. 1992; Forster and Ogata 1998; Luzzana et al. 1998; Abidi and Khan 2009). Nutrient and energy digestibility, amino acid profile and energy content may also alter the amino acid requirements (Simmons et al. 1999; De Silva et al. 2000).

Isoleucine requirement of fingering *C. catla* in the present study (3.42-3.58% dietary protein) is higher than the requirement reported by Ravi and Devaraj (1991) for fry stage of this fish (2.35% dietary protein). Ravi and Devaraj (1991) reported the isoleucine requirement by subjecting the weight gain data to broken-line regression analysis which has been reported to underestimate the requirement (Shearer 2000). However, in this study, the isoleucine requirement is worked out on the basis of quadratic regression analysis indicating high R^2 values (Fig 1, 2, 3, 4 and 5). Adoption of these statistical models may influence the estimate of the isoleucine requirements in both the studies. Moreover, the above low isoleucine requirement reported by Ravi and Devaraj (1991) is based on subjecting the weight gain data to regression analysis. Whereas in this study, the isoleucine requirement is based on subjecting the sensitive growth parameters such as protein productive value, isoleucine retention efficiency, RNA/DNA ratio and carcass protein to quadratic regression analysis in addition to growth. In addition to these, the different dietary protein levels adopted in this study (33% dry diet) and that reported by Ravi and Devaraj (1991; 40% dry diet) might also be responsible for the variation in the isoleucine requirements of *C. catla*. A huge difference in isoleucine requirement estimates in both the studies is evident when requirements are expressed as % protein, on the other hand the same difference become minor indeed when requirements are expressed as % dry diet (1.13-1.18 versus 0.94). Hence, a lower dietary protein level in this study relative to that used by Ravi and Devaraj may also and simply explain higher


T-9097

isoleucine requirement estimates.

Determination of essential amino acid requirements in studies using the dose-response approach requires addition of large amount of crystalline amino acids to obtain graded-level of test amino acid. The incorporation, however, often leads to depressed growth performance and biased amino acid requirement estimates because of crystalline amino acid leaching, different absorption kinetics of crystalline amino acids and poor diet palatability (Ambardekar et al. 2009). Coating of crystalline amino acids can reduce the solubility and non-synchronous absorption of free amino acid relative to the protein-bound ones (Segovia-Quintero and Reigh 2004; Zhou et al. 2012). Ravi and Devaraj (1991) have used the crystalline L-amino acids in unbound form which might have hampered the amino acid utilization because of reduced gut retention. However, in this study, the crystalline L-amino acids in the experimental diets were coated by casein and gelatin which increases the retention time of the amino acid in the gut leading to more efficient utilization of the ingested amino acids.

In this study, absolute weight gain was found to improve with the increase in concentration of dietary isoleucine up to 1.25% ($I_{1.25}$). Further inclusion of isoleucine ($I_{1.5}$-$I_{1.75}$) in diets led to reduction in growth of the fish. Similarly, protein productive value was found to improve with the increased inclusion of dietary isoleucine from 0.5-1.25% ($I_{0.5}$-$I_{1.25}$). However, a reduction in protein productive value at higher levels of dietary isoleucine ($I_{1.5}$-$I_{1.75}$) was recorded. This reduction in growth performance at higher levels of dietary isoleucine may be attributed to amino acid toxicity. It has also been reported that excessive levels of amino acids may become toxic and have an adverse effect on growth because the disproportionate amount of one amino acid affects the absorption and utilization of other amino acids (Murthy and Varghese 1996a). The major proportion of the limiting amino acids is used for protein synthesis while amino acid in excess will be more available for oxidation (Gahl et al. 1996) which may be the cause of growth depression at higher levels of dietary isoleucine.

Nutritional and metabolic interactions among the branched-chain amino acids isoleucine, leucine and valine have been reported for various warm blooded animals,

including man (Hambraeus et al. 1976); poultry (De'Mello and Lewis 1971, Smith and Austic 1978); rat (Harper et al. 1970) and the pig (Oestemer et al. 1973). Data on interactions among branched-chain amino acids in fish are not clear-cut and are inconsistent among species (Yamamoto et al. 2004). Chance et al. (1964) reported that isoleucine requirement in chinook salmon was influenced by dietary leucine and that excess dietary isoleucine reduced growth rates when leucine was deficient. In this study, interactions of isoleucine with the other two branched-chain amino acids were not studied.

The RNA/DNA ratio was found to increase with the increased inclusion of dietary isoleucine up to 1.25% ($I_{1.25}$). Further inclusion of isoleucine ($I_{1.5}$-$I_{1.75}$) resulted in declining RNA/DNA ratio in muscle tissue. Somatic indices including HSI, VSI and CF were also affected by varying concentrations of isoleucine. The HSI value was higher for the group fed the lowest level of dietary isoleucine ($I_{0.5}$) which may probably be due to excess accumulation of fat in liver. Farhat and Khan (2013a) have also reported that the higher value of HSI in fish fed at higher levels of dietary lysine might be due to deposition of fat in liver. A negative correlation in VSI with the increasing concentrations of dietary isoleucine was noted up to 1.25% ($I_{1.25}$). Further inclusion of dietary isoleucine at 1.5 ($I_{1.5}$) and 1.75% ($I_{1.75}$) resulted in a slight increment of VSI values. The CF was also found to improve significantly with the increase in the levels of dietary isoleucine and found to be the highest at 1.25% isoleucine ($I_{1.25}$) in dry diet. Further increase of dietary isoleucine ($I_{1.5}$-$I_{1.75}$) showed a significant drop in CF. Di et al. (2009) also reported the same pattern of RNA/DNA ratio, VSI and CF in grass carp fed graded levels of isoleucine in the diet.

Deficiency of isoleucine causes loss of weight and poor feed conversion in milk fish (Borlongan and Coloso 1993); rohu (Murthy and Varghese 1996a; Khan and Abidi 2007a) and mrigal (Ahmed and Khan 2006). However, no study has addressed the other pathological signs such as spinal deformities, bilateral cataracts and caudal fin erosion due to deficiency of isoleucine in fish. Except poor growth and feed utilization efficiency, no other diet related pathological signs were recorded in this study. All fish were found to be in good health condition.

It has been reported that the farmed *C. catla* deposited significantly higher lipid contents in liver (Hassan et al. 2010). Since level of fat deposition affects carcass quality, the mobilization of these lipid reserves is essential to improve the carcass quality. As the carcass fat content of this fish was found in the range of 3.26-5.48% as reported in this study and by earlier workers (Seenappa and Devaraj 1995; Zehra and Khan 2013a), this fish is considered to be medium fatty fish as per the classification of Lambertsen (1978). Fatty fish are prone to oxidation. Oxidation of lipids not only produces rancid odours and flavours, but can also decrease nutritional quality and safety by the formation of secondary products (Frankel 1998; Hsieh and Kinsella 1989). Dietary isoleucine contributes to the improvement of the carcass quality as it increases the activity of uncoupling proteins in muscle cells which increased fat burning. Supplementing isoleucine might be a simple way to speed metabolic rate and lose body fat (Di et al. 2009; Nishimura et al. 2010). In this study, carcass fat was found to decrease linearly with the increasing concentrations of dietary isoleucine up to 1.25% ($I_{1.25}$). However, carcass protein showed a positive trend with the increasing levels of dietary isoleucine up to 1.25% ($I_{1.25}$). A similar trend of carcass fat and carcass protein have also been reported by Di et al. (2009) in grass carp fed graded levels of dietary isoleucine.

In conclusion, the quadratic regression analysis of growth parameters at 95% maximum response exhibited the optimum dietary isoleucine requirement between 1.13-1.18% dry diets, corresponding to 3.42-3.58% dietary protein and hence is recommended for fingerling *C. catla*. Data generated during the present study would be useful in formulating isoleucine balanced feeds for the intensive culture of fingerling *C. catla*.

SUMMARY

In order to determine the dietary isoleucine requirement of fingerling catla, *Catla catla* (4.25±0.15 cm, 0.61±0.04 g), six isonitrogenous (33% crude protein) and isocaloric (16.72 kJ/g gross energy) amino acid test diets containing casein, gelatin and crystalline L-amino acids with graded levels of isoleucine (0.5, 0.75, 1.0, 1.25, 1.5 and 1.75% of the dry diet) were prepared. Triplicate groups of fish were randomly stocked in eighteen 70 L indoor polyvinyl circular troughs at a density of 25 fingerlings per trough provided with a

water flow-through system (1-1.5 L/min). The experimental diets were fed to fish to apparent satiation at 08:00, 12:30 and 17:30 h for 12 weeks. Growth of the fish was found to increase with the incremental levels of isoleucine up to 1.25% of the dry diet. Quadratic regression analysis at 95% maximum response of absolute weight gain (6.18 g/fish), protein productive value (0.32), isoleucine retention efficiency (71.91%), RNA/DNA ratio (4.81) and carcass protein (15.7%) yielded the optimum isoleucine requirement in the range of 1.13-1.18% of the dry diet, corresponding to 3.42-3.58% dietary protein. Data generated in this experiment would be useful to formulate isoleucine-balanced, cost effective quality feeds for fingerling catla.

Table 1 Composition of the experimental diets

	Dietary isoleucine levels (%)					
Ingredients (g/100 g dry diet)	**0.5 ($I_{0.5}$)**	**0.75 ($I_{0.75}$)**	**1.0 (I_1)**	**1.25 ($I_{1.25}$)**	**1.5 ($I_{1.5}$)**	**1.75 ($I_{1.75}$)**
Casein[a] (fat-free)	8	8	8	8	8	8
Gelatin[b]	2.67	2.67	2.67	2.67	2.67	2.67
Dextrin	34.75	34.59	34.43	34.26	34.10	33.94
Crystalline L-amino acid mixture[c]	25.28	25.38	25.49	25.60	25.70	25.81
Corn oil	5	5	5	5	5	5
Cod liver oil	2	2	2	2	2	2
Mineral mix[d,f]	4	4	4	4	4	4
Vitamin mix[e,f]	3	3	3	3	3	3
α- Cellulose	5.31	5.36	4.42	5.47	5.53	5.58
Carboxymethyl cellulose	10	10	10	10	10	10
Total	100	100	100	100	100	100
Added crystalline isoleucine	0.02	0.27	0.52	0.77	1.02	1.27
Total analyzed isoleucine	0.51	0.73	0.98	1.23	1.47	1.73
Analyzed crude protein	33.72	33.64	33.17	32.61	32.59	33.28
Digestible energy[g] (kJ/g, dry diet)	13.79	13.76	13.74	13.72	13.69	13.67
Calculated gross energy (kJ/g, dry diet)	16.72	16.72	16.72	16.72	16.72	16.72

[a]Crude Protein (76%); [b]Crude Protein (96%); [c]amino acid mixture (g/100 g) arginine 1.605, histidine 0.461, isoleucine variable, leucine 2.187, lysine 1.619, methionine 1.075, cystine 0.758, phenylalanine 1.632, tyrosine 1.077, threonine 1.101, tryptophan 0.436, valine 1.825. alanine 1.388, aspartic acid 0.486, glutamic acid 0.393, proline 1.573, serine 0.189, glycine variable; [d]Mineral mixture (g/100 g) calcium biphosphate 13.57; calcium lactate 32.69; ferric citrate 02.97; magnesium sulphate 13.20; potassium phosphate (dibasic) 23.98; sodium biphosphate 08.72; sodium chloride 04.35; aluminium chloride. $6H_2O$ 0.0154; potassium iodide 0.015; cuprous chloride 0.010; manganous sulphate. H_2O 0.080; cobalt chloride. $6H_2O$ 0.100; zinc sulphate. $7H_2O$ 0.40; Vitamin mixture (g/100 g dry diet) choline chloride 0.500; inositol 0.200; ascorbic acid 0.100; niacin 0.075; calcium pantothenate 0.05; riboflavin 0.02; menadione 0.004; pyridoxine hydrochloride 0.005; thiamin hydrochloride 0.005; folic acid 0.0015; biotin 0.0005; alpha-tocopherol 0.04; vitamin B_{12} 0.00001; 2 g α-cellulose; [f]Halver (2002); [g]Digestible energy was calculated on the basis of physiological fuel values 18.83, 14.64 and 35.56 kJ/g for protein, carbohydrate and fat, respectively (Jauncey 1982).

Table 2 Analyzed amino acid composition of the experimental diets (%) for fingerling *C. catla*[a]

	Dietary isoleucine levels (%)					
Amino acid	**0.5 ($I_{0.5}$)**	**0.75 ($I_{0.75}$)**	**1.0 (I_1)**	**1.25 ($I_{1.25}$)**	**1.5 ($I_{1.5}$)**	**1.75 ($I_{1.75}$)**
EAAs						
Arginine	2.10	2.16	2.02	2.12	2.15	2.13
Histidine	0.70	0.71	0.69	0.70	0.72	0.69
Isoleucine	0.51	0.73	0.98	1.23	1.47	1.73
Leucine	3.06	3.11	3.10	3.12	3.11	3.12
Lysine	2.38	2.34	2.32	2.41	2.39	2.40
Methionine	1.38	1.37	1.34	1.33	1.39	1.38
Phenylalanine	2.05	2.11	2.10	2.12	2.11	2.13
Threonine	1.45	1.42	1.46	1.48	1.41	1.44
Tryptophan	0.51	0.53	0.51	0.52	0.54	0.55
Valine	2.43	2.44	2.46	2.41	2.42	2.44
NEAAs						
Cystine	0.78	0.81	0.82	0.84	0.82	0.85
Tyrosine	1.50	1.48	1.47	1.44	1.46	1.54
Alanine	1.93	1.96	1.90	1.87	1.86	1.84
Aspartic acid	1.12	1.15	1.16	1.13	1.12	1.17
Glutamic acid	2.31	2.36	2.32	2.36	2.35	2.38
Glycine	7.62	7.50	7.36	7.25	7.11	6.94
Proline	2.83	2.82	2.81	2.79	2.80	2.78
Serine	0.57	0.58	0.59	0.55	0.59	0.54

[a]Determined by Hitachi L-8800 Automatic Amino Acid Analyzer

Table 3 Growth performance of fingerling *C. catla* fed diets containing varying levels of isoleucine[a,b]

	Dietary isoleucine levels (%)						
	0.5 ($I_{0.5}$)	0.75 ($I_{0.75}$)	1.0 (I_1)	1.25 ($I_{1.25}$)	1.5 ($I_{1.5}$)	1.75 ($I_{1.75}$)	Quadratic P>F[c]
Average initial weight (g)	0.61±0.02[a]	0.61±0.05[a]	0.62±0.03[a]	0.61±0.05[a]	0.61±0.02[a]	0.60±0.08[a]	0.133
Average final weight (g)	3.07±0.05[d]	5.29±0.04[c]	6.07±0.05[b]	7.13±0.03[a]	7.11±0.02[a]	6.16±0.04[b]	0.002
Absolute weight gain (g/fish)	2.46±0.04[d]	4.68±0.07[c]	5.45±0.03[b]	6.52±0.04[a]	6.50±0.02[a]	5.56±0.05[b]	0.001
Feed conversion ratio	3.92±0.02[a]	2.11±0.04[b]	1.79±0.02[c]	1.45±0.02[d]	1.47±0.01[d]	1.70±0.02[c]	0.013
Feed intake (g/fish)	9.64±0.23[a]	9.87±0.17[a]	9.76±0.14[a]	9.45±0.16[a]	9.56±0.14[a]	9.45±0.15[a]	0.320
Protein productive value	0.12±0.01[e]	0.19±0.02[d]	0.27±0.01[c]	0.35±0.01[a]	0.34±0.02[a]	0.29±0.03[b]	0.0097
Isoleucine retention efficiency (%)	32.61±0.61[e]	58.48±0.54[d]	65.24±0.73[c]	74.13±0.51[a]	73.98±0.64[a]	67.85±0.58[b]	0.004
RNA/DNA ratio	2.29±0.01[e]	3.38±0.02[d]	4.21±0.02[c]	5.20±0.04[a]	5.11±0.03[a]	4.68±0.02[b]	0.0036

[a]Mean values of 3 replicates ± SEM; [b]Mean values sharing the same superscripts in the same row are insignificantly different (P>0.05); [c]Significance probability associated with the F statistic.

Table 4 Carcass composition (%wet basis) and somatic indices of fingerling *C. catla* fed diets containing varying levels of isoleucine[a,b]

	Dietary isoleucine levels (%)							
	Initial	0.5 ($I_{0.5}$)	0.75 ($I_{0.75}$)	1.0 (I_1)	1.25 ($I_{1.25}$)	1.5 ($I_{1.5}$)	1.75 ($I_{1.75}$)	Quadratic P>F[c]
Moisture (%)	79.81±0.54	74.19±0.52[d]	75.51±0.41[c]	76.28±0.46[b]	77.14±0.39[a]	77.81± 0.31[a]	77.43± 0.42[a]	0.0019
Protein (%)	12.27±0.13	12.74±0.05[d]	13.62±0.07[c]	15.43±0.06[b]	16.54±0.11[a]	16.48±0.09[a]	16.26±0.08[a]	0.0094
Fat (%)	3.26±0.11	4.97± 0.02[a]	4.12± 0.04[b]	3.05± 0.03[c]	2.69±0.05[d]	2.71± 0.02[d]	2.76± 0.05[d]	0.0017
Ash (%)	2.39± 0.04	2.48±0.03[a]	2.37± 0.02[b]	2.28±0.02[c]	2.26±0.02[c]	2.27± 0.03[c]	2.29± 0.02[c]	0.0011
Hepatosomatic index%	0.84±0.06	1.21±0.05[a]	0.91±0.03[b]	0.89±0.02[b]	0.87±0.02[b]	0.85±0.04[b]	0.85±0.03[b]	0.0491
Viscerosomatic index%	6.32±0.11	6.51±0.08[a]	5.74±0.11[b]	5.11±0.14[c]	4.27±0.08[e]	4.75±0.06[d]	5.38±0.02[c]	0.02
Condition factor	0.91±0.04	0.97±0.04[e]	1.23±0.07[d]	1.42±0.09[c]	1.67±0.11[a]	1.51±0.08[b]	1.44±0.06[c]	0.013

[a]Mean values of 3 replicates ± SEM; [b]Mean values sharing the same superscripts in the same row are insignificantly different (P>0.05); [c]Significance probability associated with the F statistic.

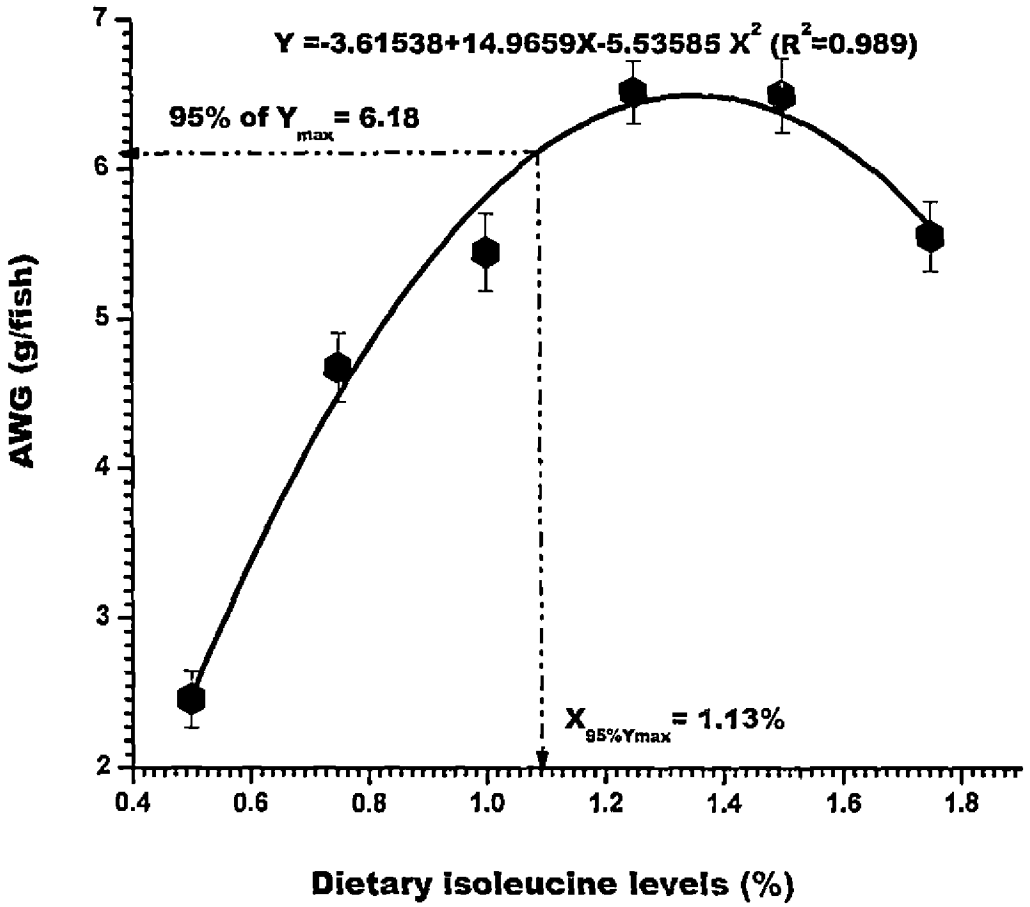

Fig. 1 Quadratic relationship of dietary isoleucine to absolute weight gain

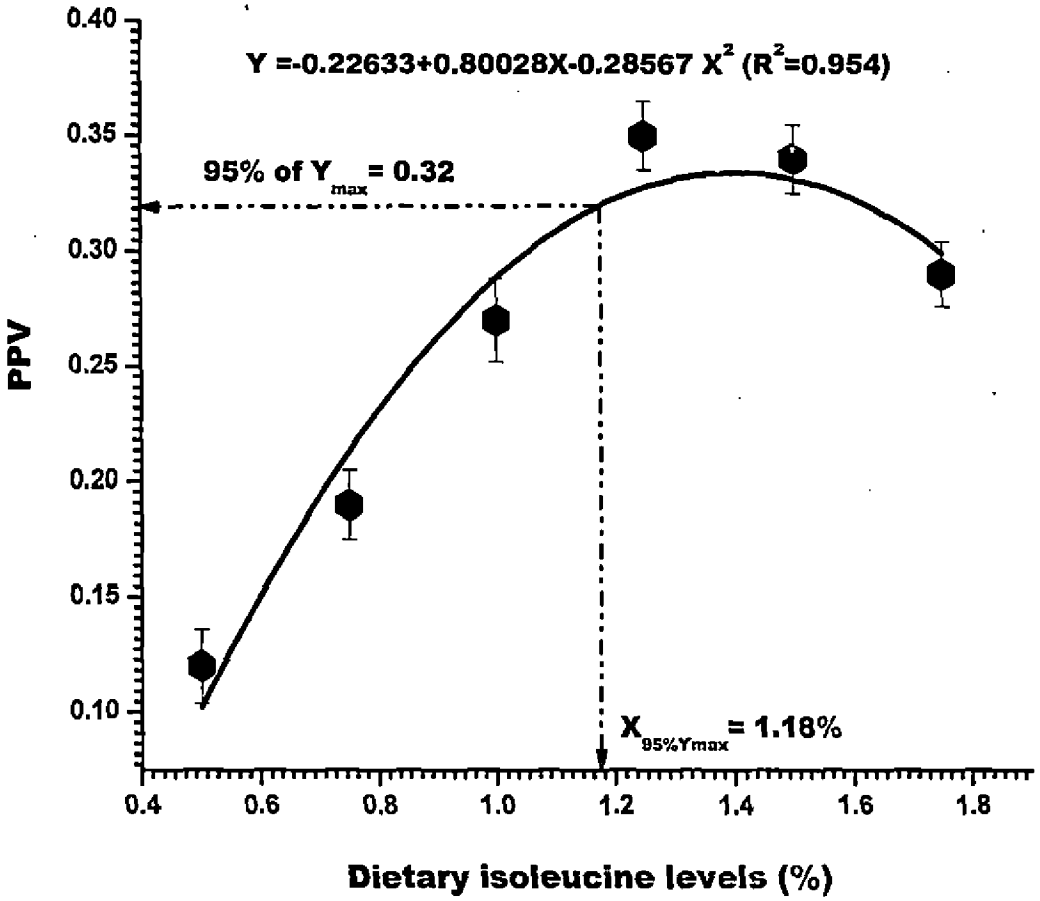

Fig. 2 Quadratic relationship of dietary isoleucine to protein productive value

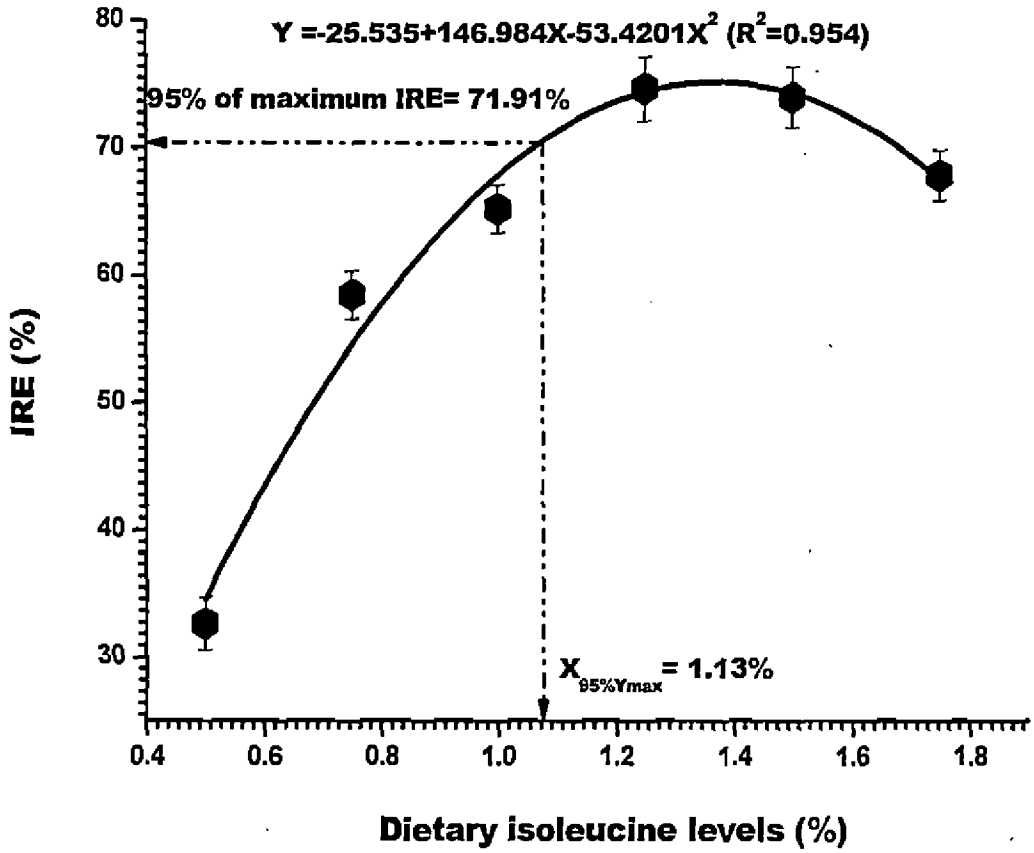

Fig. 3 Quadratic relationship of dietary isoleucine to isoleucine retention efficiency

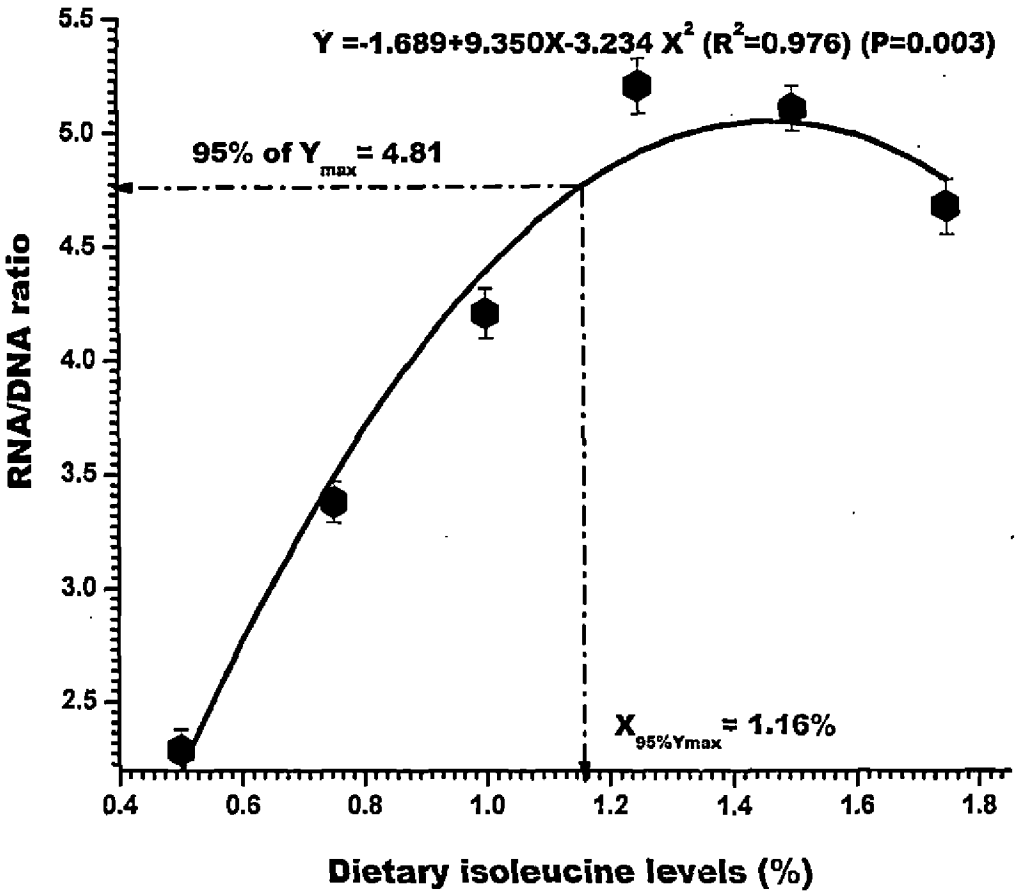

Fig. 4 Quadratic relationship of dietary isoleucine to RNA/DNA ratio

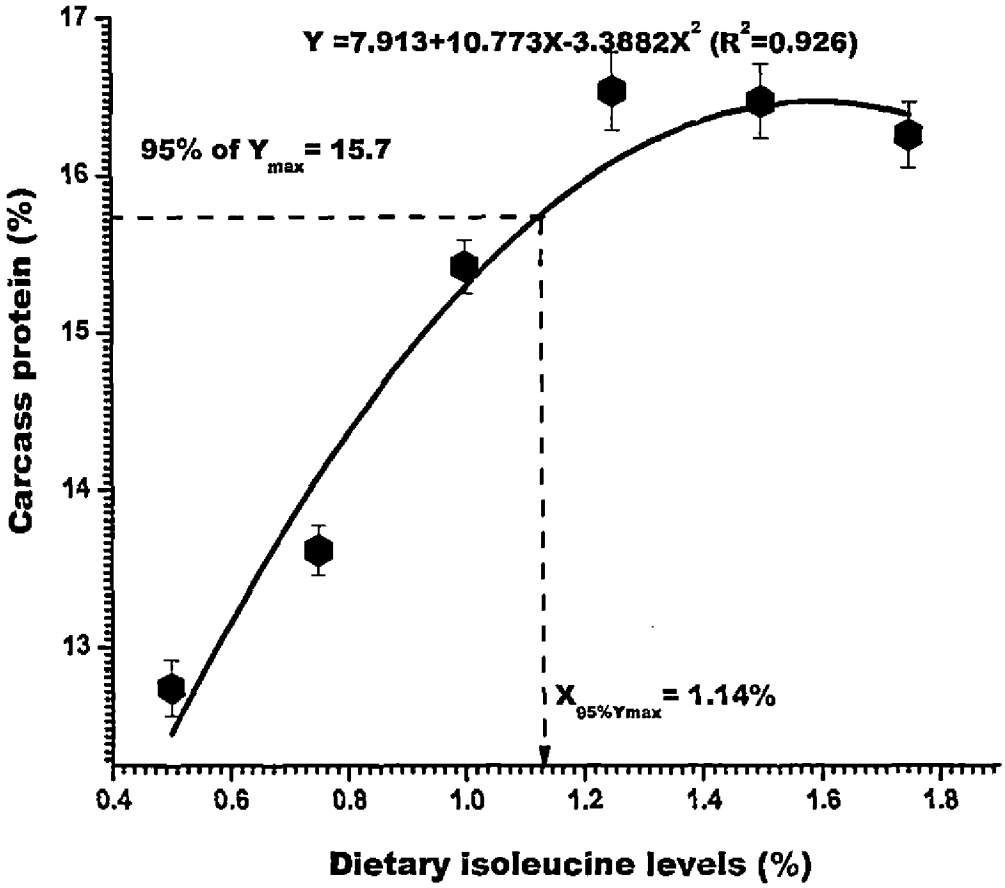

Fig. 5 Quadratic relationship of dietary isoleucine to carcass protein

CHAPTER 4

CHAPTER 4

DIETARY VALINE REQUIREMENT OF FINGERLING *CATLA CATLA* (HAMILTON) BASED ON GROWTH, PROTEIN PRODUCTIVE VALUE, VALINE GAIN AND CARCASS COMPOSITION

INTRODUCTION

For the commercial viability of any intensively cultured fish species, it is of essence to develop nutritionally balanced quality feeds. In order to produce quality feeds for fish, there is need to generate data on essential amino acid requirements of fish. Valine is one of the indispensable amino acid which is involved in many metabolic activities. It is required for the repair and growth of tissues, and also for the maintenance of nitrogen balance in body (Gore and Wolfe 2003). Valine plays very important roles in certain biochemical reactions and in the growth of monogastric and preruminant terrestrial animals. It is involved in protein synthesis, synthesis of the amine neurotransmitters serotonin and the catecholamines dopamine and norepinephrine, which are derived from the aromatic amino acids tryptophan, phenylalanine, and tyrosine, the production of energy and the compartmentalisation of glutamate (Fernstrom 2005). Valine, along with the other two branched chain amino acids leucine and isoleucine share a common membrane transport system and enzymes for their transamination and irreversible oxidation (Champe and Harvey 1987). Valine produces propinoyl CoA through catabolism which is converted to the tricarboxylic acid cycle intermediate succinyl-CoA. This amino acid can, therefore, be used to synthesize glucose (Voet and Voet 1995). It has also been reported that valine could increase fish growth through increasing digestive and absorptive capacity and influencing the balance of intestinal microflora (Dong et al. 2013). Owing to its nutritional importance, inclusion of optimum amount of dietary valine is warranted.

The valine requirements have been worked out for various fish species such as chinook salmon, *Oncorhynchus tshawytscha* (Chance et al. 1964); channel catfish, *Ictalurus punctatus* (Wilson et al. 1980); rainbow trout, *O. mykiss* (Ogino 1980; Bae et al. 2012); Mossambique tilapia, *Oreochromis mossambicus* (Jauncey et al. 1983); white

sturgeon, *Acipenser transmontanus* (Ng and Hung 1995); rohu, *Labeo rohita* (Murthy and Varghese 1997a; Abidi and Khan 2004a); red sea bream, *Chrysophrys major* (Forster and Ogata 1998; Rahimnejad and Lee 2013); Atlantic salmon, *Salmo salar* (Rollin 1999), mrigal, *Cirrhinus mrigala* (Benakappa and Varghese 2003; Ahmed and Khan 2006) and Jian carp, *Cyprinus carpio* (Dong et al. 2013).

Although data on the valine requirement of fry *C. catla* is available (Ravi and Devaraj 1991), information on the valine requirement of fingerling stage of this fish is completely lacking. The above reported valine requirement of fry *C. catla* is based on weight gain only which may be due to deposition of fat and moisture rather than body protein deposition and may not predict true growth (Cowey 1992). Hence, this study was undertaken to find out the dietary valine requirement of fingerling *C. catla* in relation to growth, feed conversion, protein productive value, valine gain and carcass composition.

MATERIALS AND METHODS

Experimental diets

Seven isonitrogenous (33% crude protein) and isocaloric (16.72 kJ/g gross energy) amino acid test diets (V1, V2, V3, V4, V5, V6 and V7) containing casein (fat-free), gelatin and crystalline L-amino acids with graded levels of valine (0.5, 0.7, 0.9, 1.1, 1.3, 1.5 and 1.7% of dry diet) were formulated. The levels of valine in the amino acid test diets were fixed on the basis of information available on other Indian major carps (Abidi and Khan 2004a; Ahmed and Khan 2006; NRC 2011). Amino acid composition of the experimental diets was emulated to that of 33% whole chicken egg protein excluding the test amino acid valine. The composition of the basal diet is given in Table 1. The amount of valine contributed by the casein and gelatin in basal diet (V1) was 0.46 and 0.04%, respectively. The amount of valine was increased at the expense of glycine, on protein to protein basis to attain the intended concentrations of dietary valine. Amino acid analysis of diets revealed the L-valine content to be 0.51, 0.69, 0.91, 1.12, 1.31, 1.49 and 1.71% of the dry diet. The analyzed amino acid composition of the basal diet is presented in Table 2. Method of preparation of experimental diets has been discussed under the General Methodology section (pages 9-10).

Experimental design and feeding trial

Source of the fish, their acclimation and details of the general experimental design has already been discussed under the General Methodology section (page 8).

Fingerling *C. catla* (3.50±0.15 cm, 0.63±0.04 g) were taken from the above acclimated fish lot and stocked in 70 L circular polyvinyl troughs (water volume 55 L) fitted with a continuous water flow-through (1-1.5 L/min) system in triplicate groups at the rate of 25 fish per trough for each dietary treatment. Fish were fed test diets in the form of dry crumbles (500 µm) to apparent satiation thrice daily at 08:00, 12:30 and 17:30h. Initial and weekly weights were recorded on a top-loading balance (Precisa 120A; 0.1 mg sensitivity, Oerlikon AG, Zurich, Switzerland) after anaesthetizing the fish with tricaine methane sulfonate (MS-222; 100 µg/ml). Fish were deprived of feed on the day they were weighed. The feeding trial lasted for 12 weeks. Faecal matter was siphoned before every feeding. Water quality indices were recorded following standard methods (APHA 1992). The average water temperature, dissolved oxygen, free carbon dioxide, pH, total ammonia nitrogen, nitrites and total alkalinity based on daily measurements, were 25.9±1.2°C, 6.7±0.7 mg/L, 8.2±1.8 mg/L, 7.5±0.3, 0.31±0.05 mg/L, 0.05±0.01 mg/L and 79.4±2.38 mg/L, respectively.

Sample collection and chemical analyses

At the beginning of the feeding trial, 60 fish were randomly sampled, killed after anaesthetizing with MS-222 (100 µg/ml) and pooled. Six subsamples of the pooled sample were analyzed for initial fish carcass composition. At the end of the experiment, 20 fishes from each replicate of dietary treatment (n=20 x 3) were randomly collected, sacrificed with an overdose of MS-222 and pooled separately. Three subsamples of each replicate of the pooled samples (n=3 x 3) were analyzed for final carcass composition. Proximate composition of casein, gelatin, experimental diets, and initial and final carcass was estimated using standard methods (pages 10-11). Gross energy content was determined on a Gallenkamp Ballistic Bomb Calorimeter as per the method described on page 12. Amino acid analysis of casein, gelatin, experimental diets, initial and final fish carcass was done using an automatic amino acid analyzer as detailed earlier (page 12).

Evaluation of growth parameters

Calculation of various growth parameters was made according to the standard definitions as described under General the Methodology section (pages 13-14).

Statistical analyses

Statistical analyses of growth data were done using procedures as detailed earlier on page 14.

RESULTS

Growth performance

Absolute weight gain (AWG, g/fish), feed conversion ratio (FCR), protein productive value (PPV) and valine gain (VG, mg/fish) of fingerling *C. catla* fed varying levels of dietary valine are reported in Table 3. Best growth performance (AWG; 7.51 g/fish, FCR; 1.47, PPV; 0.34 and VG; 50.07 mg/fish) was attained by the groups fed at 1.12% dietary valine (V4). These parameters remained insignificantly different ($P>0.05$) in group receiving diets containing higher levels of valine (V5 and V6). However, further increment in valine concentrations at 1.71% of dry diet (V7) depressed growth in terms of above parameters. Feed intake was found to increase as the dietary concentrations of valine increased up to 1.12% (V4). However, fish fed diets V5, V6 and V7 did not show significant changes ($P>0.05$) in feed intake (Table 3). Survival was unaffected by the dietary valine concentrations and no fish died during the length of the feeding trial.

In order to generate more precise information on valine requirement of fingerling *C. catla*, AWG, PPV and VG were subjected to quadratic regression analysis at 95% of maximum response. Quadratic regression analysis of AWG against dietary valine concentrations exhibited the valine requirement at 1.04% dry diet, corresponding to 3.15% dietary protein (Fig. 1). The relationship being:

$$Y_{AWG} = -4.45 + 18.152\ X - 6.89\ X^2\ (R^2 = 0.979);\ X_{95\%Ymax} = 1.04\%$$

Quadratic regression analysis of PPV data against dietary valine concentrations reflected the valine requirement at 1.03% dry diet, corresponding to 3.12% dietary protein (Fig. 2). The relationship of PPV to dietary valine levels is described by following equation:

$$Y_{PPV} = -0.245 + 0.901X - 0.342X^2 \ (R^2 = 0.963); \ X_{95\%Ymax} = 1.03\%$$

Similarly quadratic regression analysis of VG data against dietary valine concentrations revealed the valine requirement at 1.04% dry diet, corresponding to 3.15% dietary protein (Fig. 3). The relationship of VG to dietary valine levels is described by the equation as:

$$Y_{VG} = -44.3409 + 146.0017X - 55.86\ X^2 \ (R^2 = 0.981); \ X_{95\%Ymax} = 1.04\%$$

Carcass composition

Carcass composition of fingerling *C. catla* was also significantly ($P<0.05$) affected by the varying concentrations of dietary valine (Table 4). Carcass protein was found to be highest (16.21%) in fish fed diet containing 1.12% valine (V4). Quadratic regression analysis at 95% maximum response of carcass protein ($Y_{carcass\ protein} = 6.85 + 13.73X - 4.979X^2$, $R^2 = 0.982$) against dietary valine concentrations exhibited the valine requirement at 0.98% dry diet. Carcass fat showed increasing trend with the increasing levels of dietary valine (V1-V7) whereas moisture content was found to decrease with the increasing concentrations of dietary valine (V1-V7). No significant differences ($P>0.05$) were found in carcass ash among the fish fed diets with varying levels of valine. The relationship of carcass fat and moisture content to the dietary valine is expressed by the linear equations which are $Y_{carcass\ fat} = 2.423 + 1.380X$ ($R^2 = 0.872$) and $Y_{moisture\ content} = 80.581 - 4.823X$ ($R^2 = 0.993$), respectively.

Dietary valine requirement

Quadratic regression analysis of AWG, PPV, VG and carcass protein against dietary valine concentrations reflected the requirement at 1.04, 1.03, 1.04 and 0.98% dry diet, respectively. Based on the average value of the requirements reflected by above

parameters, valine requirement of fingerling *C. catla* was found to be 1.02% dry diet, corresponding to 3.09% dietary protein.

DISCUSSION

Successful fish culture depends upon the provision of feeds containing adequate and appropriate balance of nutrients to permit the most efficient growth and to maintain the health of the animal under given circumstances (Cho and Bureau 1995). Optimizing the amino acid supply in tune with the requirements and improving protein utilization for body protein growth with limited impacts on the environment in terms of nutrient loads is a generic imperative in all animal production systems and, therefore, exploratory studies on amino acid flux, inter-organ distribution and particularly of muscle protein synthesis, growth and degradation and the underlying mechanisms as affected by dietary factors are warranted (Kaushik and Seiliez 2010).

Valine is considered to be potentially limiting after lysine, methionine, threonine, and tryptophan (Barea 2009) in many plant protein sources such as soybean meal (Berres et al. 2010), corn meal (Berres et al. 2010) and chickpea meal (Hemeda and Mohamed 2010). The efficient use of valine-limiting protein in feed depends on the determination of accurate quantitative dietary valine requirements for candidate species. Based on quadratic regression analysis of growth indices, valine requirement of fingerling *C. catla* in this study was found to be 1.02% of the dry diet that corresponds to 3.09% of dietary protein. This value (3.09% of dietary protein) is higher than the requirement reported for mozambique tilapia, 2.2% (Jauncey et al. 1983); red sea bream, 2.5%, Japanese flounder, *Paralichthys olivaceus* 2.5% (Forster and Ogata 1998); but lower than that of Japanese eel, *Anguilla japonica* 4.0% (Nose 1979); milkfish, *Chanos chanos* 3.6% (Borlongan and Coloso 1993); common carp, 3.6% (Nose 1979); white sturgeon, 3.3% (Ng and Hung 1995); rohu, 3.7% (Abidi and Khan 2004a); mrigal, 3.8-3.9% (Benakappa and Varghese 2003, Ahmed and Khan 2006) and comparable to the requirement reported for channel catfish, 2.9% (Wilson et al. 1980) and Nile tilapia, *O. niloticus* 2.8% (Santiago and Lovell 1988) of the dietary protein.

The valine requirement varies from 0.7 to 3.8% of dietary protein among the

species (NRC 2011). It has been suggested that the wide variations in amino acid requirements of fish was probably due to differences in fish size and age (Forster and Ogata 1998), feeding levels (Chiu et al. 1988), quality of the diet used, laboratory conditions (Lall and Anderson 2005), and mainly due to species differences (Akiyama et al. 1997). Digestibility, amino acid profile and energy content may also bring about variable effects in amino acid requirement studies (Simmons et al. 1999; De Silva et al. 2000). Use of response variables such as weight gain, protein deposition, feed conversion ratio, RNA/DNA ratio and blood parameters may be the reason for the differences in the amino acid requirements of varying species of fish. Some of the discrepancies observed may also be due to the differences in the composition of the specific dietary protein (Rodehutscord et al. 1997). Use of diverse response fitting models may also result in different estimates of valine requirements among the fish species.

The valine requirement of fingerling *C. catla* (1.02% dry diet, corresponding to 3.09% of dietary protein) determined in this study is lower than the requirement (1.42% dry diet, corresponding to 3.55% of dietary protein) reported by Ravi and Devaraj (1991) on the fry stage of this fish. The differences in the valine requirement in both the studies may be due to the variation in fish size as fish under study was in fingerling stage requiring lower dietary nutrient requirements for the metabolical and physiological activities than the fry stage of the fish in the study conducted by Ravi and Devaraj (1991). The divergence between the findings of the present trial with those of Ravi and Devaraj (1991) may likely be a consequence of differences in dietary protein. Moreover, in this study, fish were fed three times per day in contrast to twice a day feeding frequency adopted in earlier study conducted by Ravi and Devaraj (1991). This may also be the reason for the differences in the valine requirement of *C. catla*. Disparity in the valine requirement of similar species may also be due to the differences in experimental design and available dietary energy. Coating of crystalline amino acids in this study may also lead to different valine requirement. It has been reported that coating of crystalline amino acids in diet improves the utilization of crystalline amino acids by minimizing the leaching loss and alleviating the absorption rate of free amino acids to form a more balanced amino acid pool beneficial for protein synthesis (Murai et al. 1982; Zhang 2007). Ravi and Devaraj (1991) have used uncoated crystalline amino acids which were

probably not properly utilized and hence led to higher estimate of valine requirement. However, crystalline amino acids used in this study were first coated with some amount of carboxymethyl cellulose followed by casein and gelatin which allowed their controlled release in the gut leading to optimized estimate of valine requirement.

In this study, absolute weight gain of fingerling *C. catla* was found to improve with the increase in valine concentrations up to 1.12% of the dry diet (V4). No improvement in weight gain was recorded in fish fed diets containing valine at 1.31% (V5) and 1.49% (V6) of the dry diet. However, fish fed on 1.71% valine diet (V7) showed reduced gain in weight. This reduction in growth at surfeit level of valine (V7) is presumably due to the toxic effects as reported by Choo et al. (1991) and stress caused by excess amount of amino acid in the body of the fish leading to extra energy expenditure toward deamination and excretion of the same (Walton 1985). The pattern of growth in fish fed diet containing higher level of dietary valine in this study is similar to those obtained on other carps such as *C. cirrhosus* (Benakappa and Varghese 2003), *L. rohita* (Abidi and Khan 2004a), *C. mrigala* (Ahmed and Khan 2006) and *C. carpio* (Dong et al. 2013).

Growth depressing effects from dietary supplements of excess branched-chain amino acids are most marked in diets that are deficient in one of the branched-chain amino acids (Harper et al. 1970). In this study, growth depression in fish fed diet containing higher level of dietary valine (V7) might not be due to the antagonistic effects of branched-chain amino acids but from toxicity of the excess valine itself. Since levels of isoleucine and leucine in the experimental diets were fixed at 1.18% dry diet (Zehra and Khan 2013b) and 1.58% dry diet (unpublished data from our laboratory) in this study, the possibility of interaction of valine with the other two branched-chain amino acids, isoleucine and leucine which are in balanced amounts may be ruled out.

Feed intake has been regulated by dietary amino acids inclusion. Feed intake gradually increased with the increasing dietary valine levels from 0.51-1.12% (V1-V4), thereafter, remained constant. The reduction in feed intake at lower levels of valine mainly at 0.51 (V1) and 0.69% (V2) may be due to amino acid imbalance. Halver and

Shanks (1960) have also noted a curbed feed intake, and a retardation of growth in sockeye salmon fed rations deficient in indispensable amino acids. It has been widely documented that a reduction in feed intake may be regarded as the primary factor responsible for depressed growth in fish fed diets deficient in amino acid (Ahmed and Khan 2006, Dong et al. 2013). This is also confirmed by our observation.

Bureau et al. (2000) found that weight gain of fish is associated with the accretion of protein. In the present study, fish fed basal diet (V1) showed the lowest carcass protein and protein productive value. These parameters were found to improve with the increased inclusion of dietary valine up to 1.12% (V4) indicating that valine enhanced the protein utilization of fish (Dong et al. 2013). In this study, no improvement in carcass protein was recorded at surfeit levels of dietary valine (V5-V7) indicating that excess valine could not be utilized for carcass protein synthesis and got deaminated. Quadratic regression analysis at 95% maximum response of carcass protein and PPV against dietary valine concentrations yielded the valine requirement at 0.98 and 1.03% dry diet, corresponding to 2.97 and 3.12% dietary protein, respectively.

The most frequent indicator of essential amino acid deficiencies is impaired growth and feed utilization but several specific pathological signs such as skeletal deformities in rainbow trout (Walton et al. 1986), bilateral cataracts in salmonids (Walton et al. 1982; Breck et al. 2003) and caudal fin erosion in rainbow trout (Walton et al. 1984) have been recorded under experimental conditions which may affect commercial fish farming. However, except poor growth and feed conversion efficiency, no other nutritional pathologies were recorded in this study.

Based on quadratic regression analysis of AWG, PPV, VG and carcass protein against varying levels of dietary valine, it is recommended that inclusion of valine at 1.02% of dry diet that corresponds to 3.09% dietary protein would be useful in formulating valine-balanced commercial feeds for the mass culture of fingerling *C. catla*.

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SUMMARY

A 12-week feeding-trial was conducted to determine the dietary valine requirement of fingerling *Catla catla* (3.50±0.15 cm, 0.63±0.04 g). Seven casein-gelatin based diets (33% crude protein; 16.72 kJ/g gross energy) containing graded levels of valine (0.51, 0.69, 0.91, 1.12, 1.31, 1.49 and 1.71% dry diet) were fed to triplicate groups of fish to apparent satiation at 08:00, 12:30 and 17:30 h. Absolute weight gain (AWG), protein productive value (PPV), valine gain (VG), feed conversion ratio (FCR) and carcass protein improved significantly ($P<0.05$) with the increasing concentrations of dietary valine from 0.51 to 1.12%. Quadratic regression analysis of AWG, PPV, VG and carcass protein against varying levels of dietary valine at 95% maximum ($Y_{95\%max}$) response yielded the valine requirement at 1.04, 1.03, 1.04 and 0.98% of dry diet, respectively. Based on above analysis, it is recommended that inclusion of valine at 1.02% of dry diet, corresponding to 3.09% dietary protein is optimum in formulating valine-balanced feeds for fingerling *C. catla*.

Table 1 Composition of the basal diet

Ingredients	%dry diet
Casein[a] (vitamin and fat-free)	6.8
Gelatin[b]	2.27
Dextrin	36.43
Amino acid mixture[c]	25.64
Corn oil	5
Cod liver oil	2
Mineral mix[d,f]	4
Vitamin mix[e,f]	3
α- Cellulose	4.86
Carboxymethyl cellulose	10
Total	100
Analyzed crude protein	32.87
Digestible energy[g] (kJ/g, dry diet)	13.75
Calculated gross energy (kJ/g, dry diet)	16.72

[a]Crude Protein (76%); [b]Crude Protein (96%); [c]Amino acid mixture (% dry diet) arginine 1.479, histidine 0.403, isoleucine 0.781, leucine 0.850, lysine 1.428, methionine 1.003, cystine 0.75, phenylalanine 1.52, tyrosine 0.973, threonine 1.019, tryptophan 0.34, valine 0, alanine 1.26, aspartic acid 0.324, proline 1.275, serine 0.071, glycine 9.093; (Loba Chemie, India); [d]Mineral mixture (g/100 g) calcium biphosphate 13.57; calcium lactate 32.69; ferric citrate 02.97; magnesium sulphate 13.20; potassium phosphate (dibasic) 23.98; sodium biphosphate 08.72; sodium chloride 04.35; aluminium chloride. $6H_2O$ 0.0154; potassium iodide 0.015; cuprous chloride 0.010; manganous sulphate. H_2O 0.080; cobalt chloride. $6H_2O$ 0.100; zinc sulphate. $7H_2O$ 0.40; [e]Vitamin mixture (g/100 g dry diet) choline chloride 0.500; inositol 0.200; ascorbic acid 0.100; niacin 0.075; calcium pantothenate 0.05; riboflavin 0.02; menadione 0.004; pyridoxine hydrochloride 0.005; thiamin hydrochloride 0.005; folic acid 0.0015; biotin 0.0005; alpha-tocopherol 0.04; vitamin B_{12} 0.00001; 2.0 g α-cellulose, Loba Chemie, India; [f]Halver (2002); [g]Digestible energy was calculated on the basis of physiological fuel values 18.83, 14.64 and 35.56 kJ/g for protein, carbohydrate and fat, respectively (Jauncey 1982).

Table 2 Analyzed amino acid composition of the basal diet for fingerling *C. catla*[a,b]

Amino acid	%dry diet
EAAs	
Arginine	2.13±0.04
Histidine	0.71±0.02
Isoleucine	1.17±0.05
Leucine	1.57±0.04
Lysine	2.37±0.03
Methionine	1.34±0.01
Phenylalanine	2.04±0.01
Threonine	1.44±0.02
Tryptophan	0.53±0.01
Valine	0.51±0.01
NEAAs	
Cystine	0.80±0.01
Tyrosine	1.50±0.02
Alanine	1.91±0.03
Aspartic acid	1.15±0.02
Glycine	9.78±0.05
Proline	2.73±0.02
Serine	0.59±0.01

[a]Determined by Hitachi L-8800 Automatic Amino Acid Analyzer; [b]Mean values of 5 replicates±SEM.

Table 3 Growth performance of fingerling *C. catla* fed diets containing varying levels of valine[a,b]

	Varying levels of analyzed valine (% dry diet)						
	0.51 (V1)	0.69 (V2)	0.91 (V3)	1.12 (V4)	1.31 (V5)	1.49 (V6)	1.71 (V7)
Average initial weight (g)	0.64±0.02[a]	0.63±0.05[a]	0.62±0.03[a]	0.63±0.05[a]	0.63±0.02[a]	0.63±0.04[a]	0.63±0.04[a]
Average final weight (g)	3.12±0.07[e]	6.01±0.12[d]	7.07±0.07[c]	8.14±0.14[a]	8.11±0.09[a]	8.06±0.13[a]	7.64±0.13[b]
Absolute weight gain (g/fish)	2.86±0.11[e]	5.38±0.15[d]	6.45±0.08[c]	7.51±0.17[a]	7.48±0.12[a]	7.43±0.13[a]	7.01±0.13[b]
Feed conversion ratio	2.99±0.03[a]	1.84±0.04[b]	1.63±0.02[c]	1.47±0.05[d]	1.51±0.03[d]	1.52±0.03[d]	1.62±0.03[c]
Feed intake (g/fish)	8.55±0.12[d]	9.89±0.11[c]	10.51±0.08[b]	11.04±0.26[a]	11.14±0.24[a]	11.29±0.21[a]	11.35±0.21[a]
Protein productive value	0.11±0.01[e]	0.24±0.01[d]	0.28±0.01[c]	0.34±0.01[a]	0.33±0.01[a]	0.33±0.02[a]	0.30±0.02[b]
Valine gain (mg/fish)	15.01±0.19[e]	35.09±0.23[d]	44.81±0.34[c]	50.07±0.46[a]	49.69±0.53[a]	48.98±0.71[a]	46.14±0.54[b]

[a]Mean values of 3 replicates ± SEM. [b]Mean values sharing the same superscripts in the same row are insignificantly different ($P>0.05$).

Table 4 Carcass composition (wet basis) of fingerling *C. catla* fed diets containing varying levels of valine[a,b]

	Varying levels of analyzed valine (% dry diet)							
	initial	0.51 (V1)	0.69 (V2)	0.91 (V3)	1.12 (V4)	1.31 (V5)	1.49 (V6)	1.71 (V7)
Moisture (%)	79.59±1.54	78.21±1.38^{a}	77.14±1.54^{a}	76.17±1.26^{ab}	75.22±1.19^{ab}	74.67±1.13^{ab}	73.16±1.21^{b}	72.36±1.21^{b}
Protein (%)	12.16±0.13	12.38±0.11^{d}	14.14±0.13^{c}	15.11±0.18^{b}	16.21±0.19^{a}	16.12±0.17^{a}	16.01±0.15^{a}	15.98±0.18^{a}
Fat (%)	3.19± 0.11	2.81± 0.02^{d}	3.39± 0.04^{c}	3.82± 0.03^{b}	4.26±0.03^{a}	4.37± 0.02a^{a}	4.43± 0.05^{a}	4.51± 0.03^{a}
Ash (%)	2.41± 0.04	2.42±0.02^{a}	2.44± 0.01^{a}	2.45±0.02^{a}	2.43±0.02^{a}	2.42± 0.03^{a}	2.45± 0.02^{a}	2.43± 0.02^{a}

[a]Mean values of 3 replicates ± SEM; [b]Mean values sharing the same superscripts in the same row are insignificantly different (P>0.05).

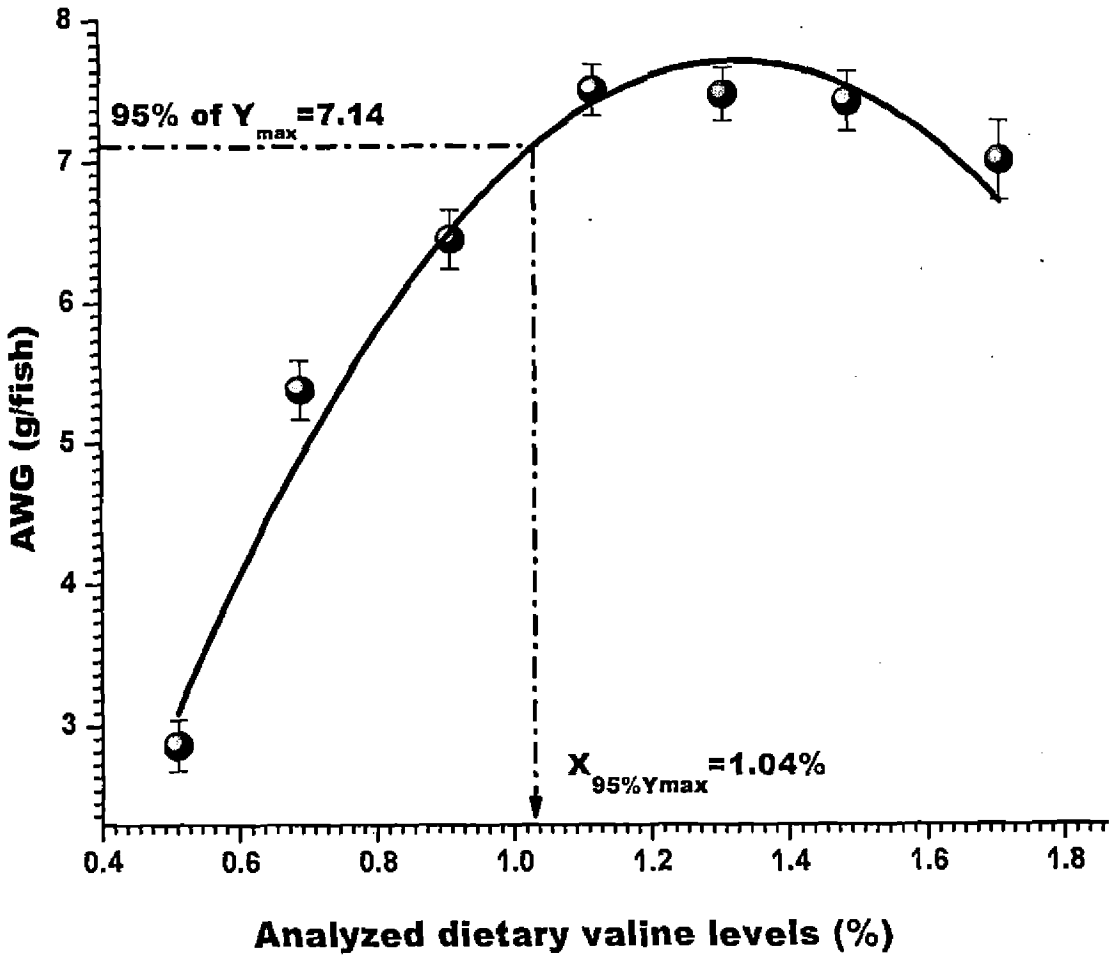

Fig. 1 Quadratic relationship of dietary valine to absolute weight gain

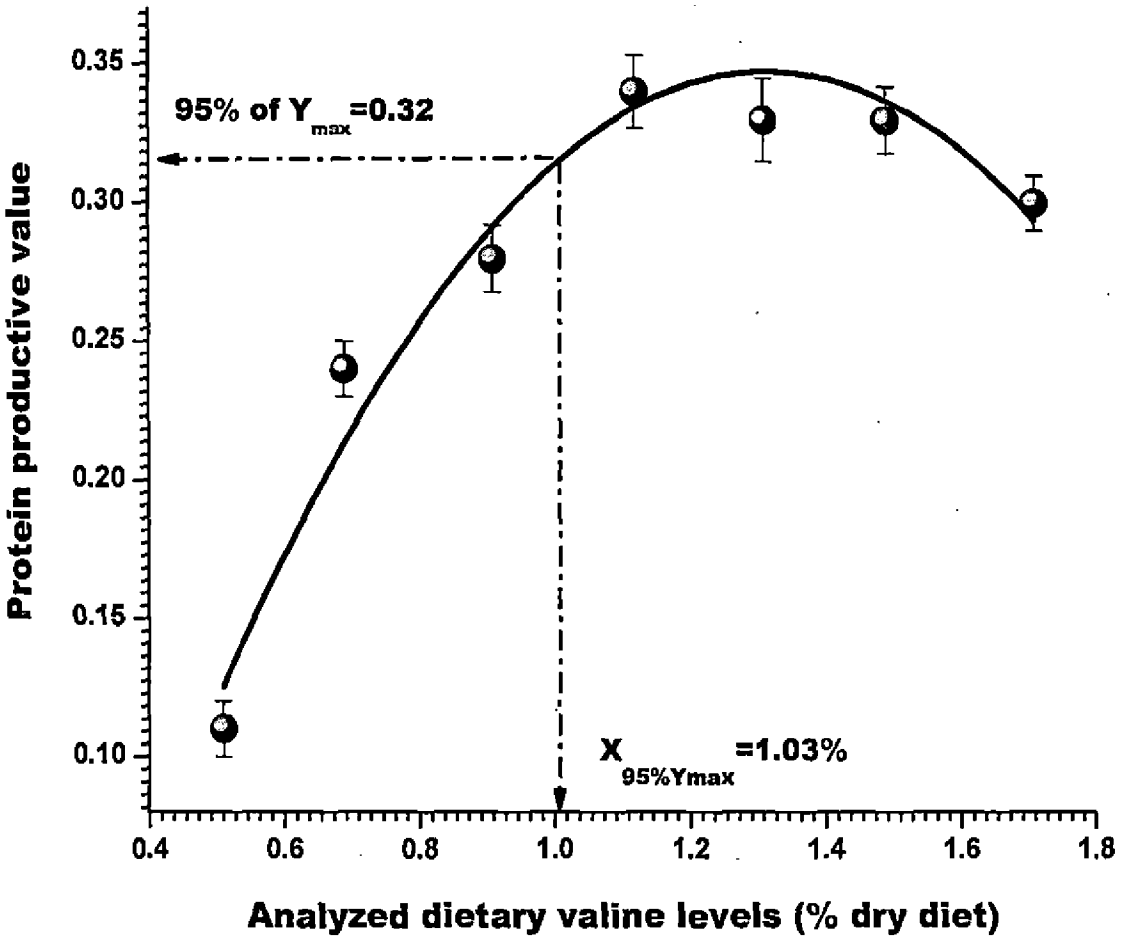

Fig. 2 Quadratic relationship of dietary valine to protein productive value

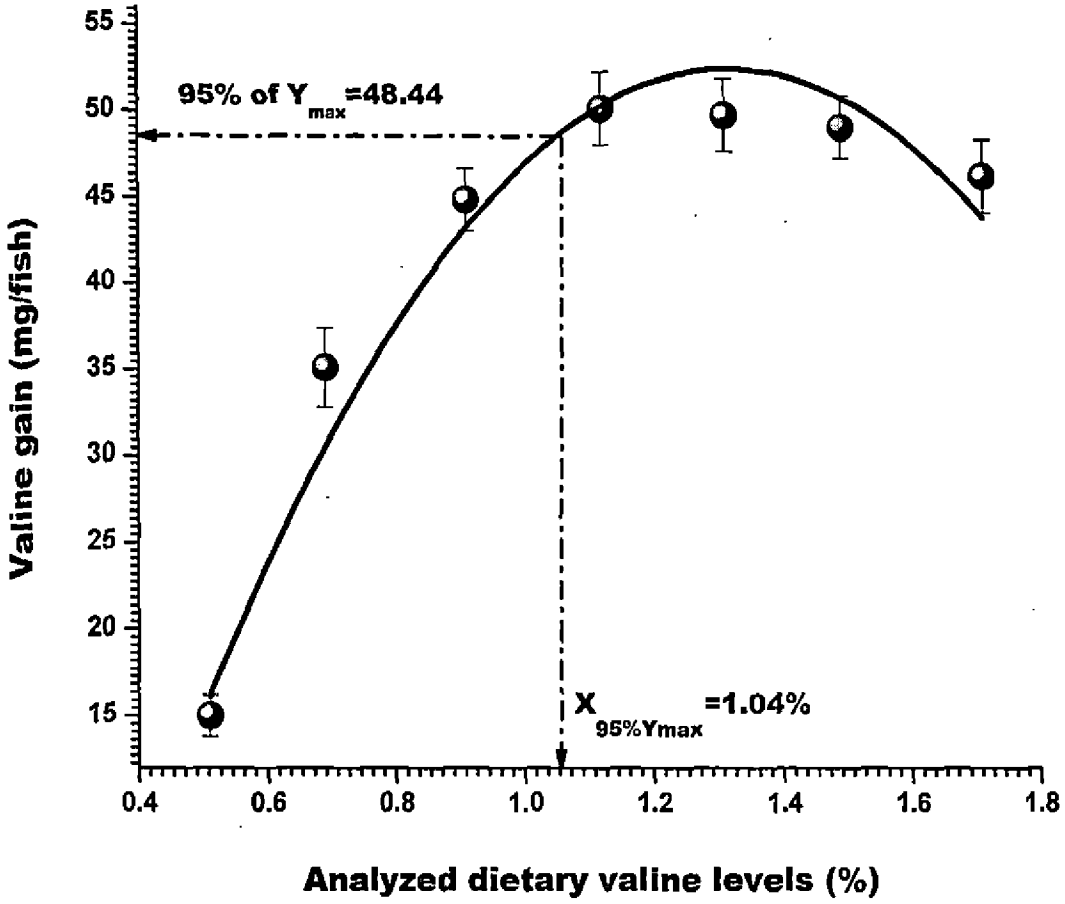

Fig. 3 Quadratic relationship of dietary valine to valine gain

CHAPTER 5

CHAPTER 5

DIETARY LEUCINE REQUIREMENT OF FINGERLING *CATLA CATLA* (HAMILTON) BASED ON GROWTH, FEED CONVERSION RATIO, RNA/DNA RATIO, LEUCINE GAIN, BLOOD INDICES AND CARCASS COMPOSITION

INTRODUCTION

The development of nutritionally adequate, cost-effective feeds for all stages of cultured fish species is of great importance to the commercial success of aquaculture. Formulation of balanced and cost-effective diets requires complete knowledge of nutritional requirements of the cultured species (Wilson 1985; Lin et al. 2013). Dietary intake of essential amino acids is required to achieve optimum growth, best feed conversion and desirable carcass quality. Leucine, a member of aliphatic side chain amino acid family is essential for normal growth and reproductive potential of the fish (Abidi and Khan 2007). It plays an important role in protein synthesis, promotes insulin release, and inhibits protein degradation (Nair et al. 1992). Leucine has also been implicated to play a signaling role in enhancing the availability of specific eukaryotic initiation factors (Anthony et al. 2000) as well as augmenting the activity of proteins involved in mRNA translation (Davis and Fiorotto 2009; Wu et al. 2010). It also supplies gluconeogenic precursors via the formation of alanine in muscle (Brooks 1987). The essential branched chain amino acid leucine amounts to about 4.6% of the total amino acids (Takala et al. 1980).

Dietary requirements of leucine have been worked out for various cultivable fish species such as chinook salmon, *Oncorhynchus tshawytscha* (Chance et al. 1964); channel catfish, *Ictalurus punctatus* (Wilson et al. 1980); rainbow trout, *O. mykiss* (Ogino 1980); Mossambique tilapia, *Oreochromis mossambicus* (Jauncey et al. 1983); white sturgeon, *Acipenser transmontanus* (Ng and Hung 1995); rohu, *Labeo rohita* (Murthy and Varghese 1997b; Abidi and Khan 2007); red sea bream, *Chrysophrys major* (Forster and Ogata 1998); European sea bass, *Dicentrarchus labrax*, gilthead seabream, *Sparus aurata* and turbot, *Scophthalmus maximus* (Kaushik 1998); Atlantic salmon, *Salmo salar*

(Rollin 1999); mrigal, *Cirrhinus mrigala* (Benakappa and Varghese 2003; Ahmed and Khan 2006) and yellow croaker, *Larimichthys crocea* (Yan et al. 2010).

Although data on leucine requirement of fry *C. catla* is available (Ravi and Devaraj 1991), information on leucine requirement for fingering *C. catla* is warranted. Therefore, this study was carried out to determine the dietary leucine requirement of fingerling *C. catla* using growth, feed conversion ratio, RNA/DNA ratio, protein gain, leucine gain and carcass composition as the sensitive parameters. Relevance of hematological indices in assessing the health status of fish has been reported by several authors (Buentello et al. 2007; Ahmed 2012a,b; Farhat and Khan 2012b). Considering the importance of hematological parameters in assessing the health status of fish in response to dietary amino acids, these tools were also utilized to estimate the dietary leucine requirement of this fish.

MATERIALS AND METHODS

Experimental diets

Six isonitrogenous (33% crude protein) and isocaloric (16.72 kJ/g gross energy) amino acid test diets using casein (fat-free), gelatin and crystalline L-amino acids with graded levels of leucine (0.75, 1.0, 1.25, 1.5, 1.75 and 2.0% dry diet) were formulated. The experimental diets were marked as L1, L2, L3, L4, L5 and L6. The levels of leucine in the amino acid test diets were fixed on the basis of information available on other Indian major carps (Murthy and Varghese 1997b; Ahmed and Khan 2006; Abidi and Khan 2007; NRC 2011). The amino acids profile of the experimental diets excluding the test amino acid leucine was simulated to that of 33% whole chicken egg protein. The composition of the basal diet is given in Table 1. Casein and gelatin served as intact protein sources and provided 0.75% leucine in the basal diet (L1). The amount of leucine was increased at the expense of glycine, on protein to protein basis to attain the intended concentrations of dietary leucine. Amino acid analysis of diets revealed the L-leucine content to be 0.73, 0.97, 1.24, 1.46, 1.74 and 1.97% of the dry diet. The analyzed amino acid composition of the basal diet is presented in Table 2. Method of preparation of

experimental diets has been discussed under General Methodology section (pages 9-10.

Experimental design and feeding trial

Source of the fish, their acclimation and details of the general experimental design has already been discussed under the General Methodology section (page 8).

Fingerling *C. catla* (3.75±0.15 cm, 0.66±0.04 g) were taken from the above acclimated fish lot and stocked in triplicate groups in 70 L circular polyvinyl troughs (water volume 55 L) fitted with a continuous water flow-through (1-1.5 L/min) system at the rate of 25 fish per trough for each dietary treatment level. Fish were fed test diets in the form of dry crumbles (500 µm) to apparent satiation thrice daily at 08:00, 12:30 and 17:30h. Initial and weekly weights were recorded on a top-loading balance (Precisa 120A; 0.1 mg sensitivity, Oerlikon AG, Zurich, Switzerland) after anaesthetizing the fish with tricaine methane sulfonate (MS-222; 100 µg/ml). Fish were deprived of feed on the day they were weighed. The feeding trials lasted for 12 weeks. Faecal matter was siphoned before every feeding. Water quality indices were monitored daily during the feeding trial and were recorded following standard methods (APHA 1992). The range of water temperature, dissolved oxygen, free carbon dioxide, pH, total ammonia nitrogen, nitrites and total alkalinity based on daily measurements, was 27.4-28.2°C, 6.4-7.2 mg/L, 5.9-9.1 mg/L, 7.3-7.5, 0.29-0.32 mg/L, 0.04-0.07 mg/L and 74.6-83.5 mg/L, respectively.

Chemical analyses

Proximate composition of casein, gelatin, experimental diets, and initial and final carcass was estimated using standard methods as detailed on pages 10-11. Gross energy content was determined on a Gallenkamp Ballistic Bomb Calorimeter as per the method described on page 12. Amino acid analysis of casein, gelatin, experimental diets, initial and final fish carcass was done using an automatic amino acid analyzer as detailed earlier (page 12). At the beginning of the feeding trial, 60 fish were randomly sampled, killed and pooled together. Six subsamples of a pooled sample were analyzed for initial carcass composition. At the end of the experiment, 20 fish from each replicate of dietary treatments were randomly collected, sacrificed with an overdose of the MS-222 and

pooled separately. Three subsamples of the pooled samples carcass composition.

Hematological analyses

At the termination of the feeding trial, blood samples were collected in heparinized vials through cardiac puncture of the fish. The blood of five fish from each replicate of the treatment group was pooled to obtain enough samples for hematological analysis. Hematocrit levels were determined by drawing fresh blood into tubes and centrifuged in a microhematocrit centrifuge at 10,000 g for 5 min (Goldenfarb et al. 1971). Red blood cell counts (RBCs) and hemoglobin (Hb) were analyzed as per the method adopted by Vani et al. (2012). In brief, 20 µl of blood was mixed with 3,980 µl of red blood cell diluting fluid (Dacies fluid) in a clean glass vial. The mixture was shaken well to suspend the cells uniformly in the solution. The cells were counted using a Neubauer hemocytometer. The blood hemoglobin content was analyzed following the cyanmethaemoglobin method using Darbkins Fluid. 20 µl of blood was mixed with 5 mL of Darbkin's working solution. The absorbance was measured using a spectrophotometer at wavelength of 540 nm. The final concentration was calculated after comparing with the standard.

Determination of RNA and DNA

RNA and DNA were determined by the method of Schneider (1957) as detailed earlier on page 13.

Evaluation of growth performance

Calculation of various growth parameters was made according to the standard definitions as described under the General Methodology section (pages 13-14).

Statistical analyses

Statistical analyses of growth data were done using procedures as detailed earlier (page 14).

RESULTS

Absolute weight gain (AWG, g/fish), feed conversion ratio (FCR), protein gain (PG, g/fish), leucine gain (LG, mg/fish) and RNA/DNA ratio of fingerling *C. catla* fed diets with different concentrations of leucine are given in Table 3. The leucine requirement of fingerling *C. catla* was obtained by quadratic regression analysis of above parameters at 95% of maximum ($Y_{95\%max}$) and minimum responses ($Y_{95\%min}$). The $Y_{95\%max}$ of AWG and $Y_{95\%min}$ of FCR yielded the leucine requirement to be 1.58 and 1.57% of dry diet, respectively. Similarly on solving the quadratic equations for PG and RNA/DNA ratio at $Y_{95\%max}$, leucine requirement was obtained at 1.59 and 1.57% dry diet, respectively. On regressing LG data against dietary leucine concentrations, the requirement was found to be 1.58% dry diet. Feed intake did not show significant differences ($P>0.05$) among the varying treatment groups (Table 3). A 100% survival was recorded in all the dietary treatments.

The quadratic equations employed for AWG (Fig. 1), FCR, PG (Fig. 2), LG (Fig. 3) and RNA/DNA ratio are $Y=-6.056+14.0766X-3.746X^2$ ($R^2=0.987$), $X_{95\%Ymax}=1.58\%$; $Y=10.6152-11.011X-3.27714X^2$ ($R^2=0.952$), $X_{95\%Ymax}=1.57\%$; $Y=-1.2956+2.5999\ X-0.6664X^2$ ($R^2=0.987$), $X_{95\%Ymax}=1.59\%$; $Y=-99.7278+195.786\ X-51.8975X^2$ ($R^2=0.990$), $X_{95\%Ymax}=1.58\%$ and $Y=-1.84157+6.67686X-1.73143X^2$ ($R^2=0.997$), $X_{95\%Ymax}=1.57\%$ dry diet, respectively.

Carcass composition of fingerling *C. catla* showed significant variations with the increase in dietary leucine levels except for carcass ash which remained almost constant (Table 4). Carcass protein was found to improve quadratically ($Y=3.09543+15.342X-4.19429\ X^2$, $R^2=0.999$) with the increasing concentrations of dietary leucine up to 1.74% (L5) but a further elevation to 1.97% (L6) could not produce any additional growth promoting effect. Carcass fat increased with the incremental levels of leucine from 0.73 (L1)-1.97% (L6) of the dry diet. However, moisture content showed a negative trend to that of the carcass fat. The relationship of carcass fat and moisture content to the dietary leucine concentrations (L1-L6) is expressed by the linear function shown below:

$Y=1.91867+1.664X$ ($R^2=0.990$), $P<0.01$ (Carcass fat)

$Y=82.46724-0.15799X$ ($R^2=0.996$), $P<0.01$ (Carcass moisture)

Table 5 revealed the effect of increasing levels of dietary leucine on hematological indices of fish. Hemoglobin (g dl^{-1}), hematocrit (%) and RBCs (10^6 x mm^{-3}) significantly ($P<0.05$) increased with the increasing concentrations of leucine up to 1.74% of the dry diet (L5) and, thereafter (L6), a decline was noted.

DISCUSSION

The leucine requirement of fingerling *C. catla* obtained by solving the quadratic equations ($Y_{95\%max}$) for absolute weight gain, feed conversion ratio, protein gain, leucine gain, carcass protein and RNA/DNA ratio was found to range between 1.57-159% dry diet, equivalent to 4.75-4.82% dietary protein which is higher than the requirement reported for other fish species including chinook salmon, *O. tshawytscha* 3.9% (Chance et al. 1964); common carp, *Cyprinus carpio* 3.3% (Nose 1979); coho salmon, *O. kisutch* 3.4% (Arai and Ogata 1993); white sturgeon, *A. transmontanus* 4.3% (Ng and Hung 1995); red seabream, 4.2%; Japanese flounder, *C. major* 3.9% (Forster and Ogata 1998); rainbow trout, *O. mykiss* 4.4% (Kaushik 1998); mrigal, *C. mrigala* 3.9% (Ahmed and Khan 2006); channel catfish, *I. punctatus* 4.5%; common carp, *C. carpio* 4.4% (NRC 2011); lower than the requirement of Atlantic salmon, *S. salar* 5.2% (Rollin 1999); yellow croaker, *L. crocea* 6.8% (Yan et al. 2010) and approximately equal to the requirement reported for rohu, *L. rohita* 4.7% (NRC 2011) of dietary protein. Different statistical models adopted may be responsible for the variations in the leucine requirement of varying species. Dietary protein level may also be attributed to differences in the leucine requirements among species. The discrepancies in the amino acids requirements may also be due to differences in fish size, age, feeding levels, flow rate, stock density and the environmental and, culture conditions adopted by different laboratories (Chiu et al. 1988; Forster and Ogata 1998; Luzzana et al. 1998; Abidi and Khan 2009). Dietary metabolizable energy, availability of dietary amino acids, antagonism and imbalance among amino acids may also be responsible for the wide

variations in amino acid requirements (Ishibashi and Ohta 1999).

Leucine requirement of fingering *C. catla* determined in this study (1.57-159% dry diet, equivalents to 4.75-4.82% dietary protein) is higher than the requirement reported by Ravi and Devaraj (1991) for fry stage of this fish (1.48% dry diet, equivalents to 3.70% dietary protein). Ravi and Devaraj (1991) reported the leucine requirement by subjecting the weight gain data to broken-line regression analysis which has been reported to underestimate the requirement (Shearer 2000). However, in this study, the leucine requirement is worked out on the basis of quadratic regression analysis which is a good fit as indicated by high R^2 values obtained for AWG (0.987), FCR (0.952), PG (0.987), LG (0.990) and RNA/DNA ratio (0.997). Adoption of these statistical models may influence the estimate of the leucine requirements in both the studies. Moreover, leucine requirement reported by Ravi and Devaraj (1991) is based on weight gain only whereas in this study, in addition to weight gain, the leucine requirement is also based on the sensitive parameters such as feed conversion ratio, protein gain, leucine gain, RNA/DNA ratio, hematological parameters and carcass composition. In addition to these, the different dietary protein levels adopted in this study (33% dry diet) and that fixed by Ravi and Devaraj (1991; 40% dry diet) might also be responsible for the variation in the leucine requirements of *C. catla*.

Dietary leucine concentrations had an impact on growth performance of different fish species (Ng and Hung 1995, Forster and Ogata 1998, Benakappa and Varghese 2003, Ahmed and Khan 2006, Abidi and Khan 2007 and Yan et al. 2010). In this study, absolute weight gain showed a quadratic response ($Y_{95\%max}$) reaching the highest value at 1.58% dietary leucine. Further inclusion of dietary leucine resulted in slight reduction in weight gain. This reduction in growth at surfeit level of dietary leucine is probably a consequence of the amino acid toxicity or dietary amino acid imbalance. The growth depression in fish fed diets containing higher level of leucine as evident in this study was also noted by earlier workers (Murthy and Varghese 1997b; Abidi and Khan 2007; Ng and Hung 1995; Forster and Ogata 1998; Benakappa and Varghese 2003; Ahmed and Khan 2006; Yan et al. 2010).

Antagonism between branched-chain amino acids generally arises in animals from an excess of leucine over isoleucine and valine because the requirement of branched chain amino acid is affected by each other (De'Mello and Lewis 1971). Choo et al. (1991) have reported that excessive leucine resulted in depressed growth and protein deposition of rainbow trout likely due to antagonism among BCAA. Excesses of leucine are extremely disruptive to utilization of isoleucine and valine, especially when these two amino acids are marginal or limiting (Smith and Austic 1978; Waldroup et al. 2002). In this study, growth reduction at surplus level of dietary leucine might not be the result of antagonistic effect of BCAA because the level of isoleucine and valine was fixed at 1.18% dry diet (Zehra and Khan 2013b) and 1.02% dry diet (unpublished data from our laboratory), respectively. Thus, the possibility of antagonism among leucine, isoleucine and valine is ruled out.

Protein synthesis and deposition are known to be the most efficient when all the required amino acids are present simultaneously at the sites of synthesis (Ng et al. 1996). In the present study, carcass protein and protein gain showed quadratic response with varying concentrations of leucine and improved up to 1.74% of the dry diet (L5). This improvement suggests that better dietary amino acid balance probably prevented the catabolism of amino acids and led to maximum protein gain at this level of dietary leucine. Carcass fat showed a positive trend with the increasing concentrations of dietary leucine. This increase in carcass fat may be because of the fact that leucine is a ketogenic amino acid, the carbon skeleton of which is converted to acetyl-CoA and acetoacetate in muscle tissue and these intermediates can be used to synthesize fatty acids (Hyun et al. 2007; Erwan et al. 2009). Ahmed and Khan (2006) have reported improvement in protein deposition up to the requirement level and a linear positive correlation in carcass fat with the increase of dietary leucine. However, Yan et al. (2010) have reported no marked variations in these parameters with the increasing concentrations of leucine.

The RNA/DNA ratio has been used as a sensitive indicator of nutritional condition in several fish species (Mustafa 1977; 1979; 1983; Mustafa and Jafri 1977; Mustafa and Mittal 1982; Mustafa and Zofair 1985; Bulow 1987; Mustafa et al. 1991; Buckley et al. 1999; Abidi and Khan 2009). Cellular RNA is essential for the

biosynthesis of protein (Clemmesen 1994). Since RNA/DNA ratio reflects the cellular ability to produce RNA and proteins, this parameter measures the potential for growth. In fish losing weight, the size of cells decreases and thus number of cells contributing to unit weight of tissue increases, enhancing the number of nuclei and contributing to increased DNA concentration. The reduced values of RNA/DNA ratio in fish fed diets containing lower levels of dietary leucine (L1-L4) in this study may be because of the above fact. On the other hand, in a weight-gaining fish, the DNA concentration becomes diluted with larger volume of cells per unit weight leading to reduction in number of cells per unit weight of the tissue. Thus, increase in cell size as a result of weight gain led to dilution of DNA per unit weight of the tissue resulting to higher RNA/DNA ratio at 1.74% leucine of the dry diet in this study. The highest RNA/DNA ratio at 1.74% dietary leucine indicates that this level might be optimum to maximize protein synthesis in fingerling of *C. catla* as evident by highest carcass protein at this level (1.74%) of dietary leucine (L5).

Blood parameters are considered as convincible indicator for the health and physiological conditions of fish (Kader et al. 2010). The count of red blood cells is quite a stable index and the animal body tries to maintain this count within the limits of certain physiological standards using various physiological mechanisms of compensation (Al-Akel et al. 2010). Low RBCs count coupled with low hemoglobin content at lower levels of dietary leucine in this study may be the result of inadequate amount of leucine available for erythropoiesis. Hematological characteristics of fingerling *C. catla* attained its peak at 1.74% (L5) dietary leucine indicating that, in addition to maximizing growth performance and protein retention, above level of dietary leucine is adequate to support the maximum Hb, Hct% and blood cell formation as well.

Generally deficiency of most essential amino acids leads to failure of weight gain and loss of appetite rather than pathological signs. The pathological signs have also been recorded in several studies. These pathological signs include spinal deformities, bilateral cataracts, caudal fin erosion (Walton et al. 1982; 1984; 1986; Breck et al. 2003). However, in this study, no such pathological signs except poor growth and feed efficiency were observed during the length of this feeding trial. All fish were found to be in healthy condition and a 100% survival was recorded among the varying treatment

groups.

On the basis of quadratic regression analysis of AWG, FCR, PG, LG and RNA/DNA ratio, the leucine requirement of fingerling *C. catla* is recommended in the range of 1.57-159% dry diet, equivalent to 4.75-4.82% dietary protein. The information generated during this study would be helpful in formulating leucine balanced feeds for the intensive culture of this fish species.

SUMMARY

This study was aimed at quantifying leucine requirement of fingerling *Catla catla* (3.75±0.15 cm, 0.66±0.04 g) by conducting a 12-week feeding trial. Six casein-gelatin based (33% crude protein, 16.72 kJ/g gross energy) amino acid test diets containing different concentrations of leucine (0.73, 0.97, 1.24, 1.46, 1.74 and 1.97% dry diet) were fed to triplicate groups of fish to apparent satiation thrice daily at 08:00, 12:30 and 17:30h. Maximum absolute weight gain (AWG, 7.45 g/fish), protein gain (PG, 1.31 g/fish), leucine gain (LG, 85.33 mg/fish), RNA/DNA ratio (4.62) and best feed conversion ratio (FCR, 1.51) were recorded at 1.74% dietary leucine. Hematological characteristics were also found to be optimum in fish fed diet with 1.74% leucine. Quadratic regression analysis at 95% maximum response of AWG, PG, LG, RNA/DNA ratio and minimum response of FCR against dietary leucine concentrations reflected the requirement at 1.58, 1.59, 1.58, 1.57 and 1.57% dry diet, respectively. Based on above results, inclusion of leucine ranging from 1.57-1.59% of the dry diet, corresponding to 4.76-4.82% dietary protein is recommended for developing leucine-balanced commercial feeds for the intensive culture of *C. catla*.

Table 1 Composition of the basal diet

Ingredients	% dry diet
Casein[a] (vitamin and fat-free)	7
Gelatin[b]	2.33
Dextrin	36.68
Amino acid mixture[c]	25.23
Corn oil	5
Cod liver oil	2
Mineral mix[d,f]	4
Vitamin mix[e,f]	3
α- Cellulose	4.75
Carboxymethyl cellulose	10
Total	100
Analyzed crude protein	33.11
Digestible energy[g] (kJ/g, dry diet)	13.86
Calculated gross energy (kJ/g, dry diet)	16.72

[a]Crude Protein (76%); [b]Crude Protein (96%); [c]Amino acid mixture (% dry diet) arginine 1.669, histidine 0.489, isoleucine 0.760, leucine 0, lysine 1.714, methionine 1.111, cystine 0.762, phenylalanine 1.689, tyrosine 1.129, threonine 1.142, tryptophan 0.444, valine 0.521; alanine 1.453; aspartic acid 0.569, glutamic acid 0.637, proline 1.724, serine 0.221 glycine 9.222 (Loba Chemie, India).[f]Halver (2002); Loba Chemie, India; [d,f]Mineral mixture (g/100g of mineral mixture) calcium biphosphate 13.57; calcium lactate 32.69; ferric citrate 2.97; magnesium sulphate 13.20; potassium phosphate (dibasic) 23.98; sodium biphosphate 08.72; sodium chloride 04.35; aluminium chloride. $6H_2O$ 0.0154; potassium iodide 0.015; cuprous chloride 0.010; manganous sulphate. H_2O 0.080; cobalt chloride. $6H_2O$ 0.100; zinc sulphate. $7H_2O$ 0.40; [e,f]Vitamin mixture (g/100 g dry diet) choline chloride 0.500; inositol 0.200; ascorbic acid 0.100; niacin 0.075; calcium pantothenate 0.05; riboflavin 0.02; menadione 0.004; pyridoxine hydrochloride 0.005; thiamin hydrochloride 0.005; folic acid 0.0015; biotin 0.0005; alpha-tocopherol 0.04; vitamin B_{12} 0.00001; 2.0 g α-cellulose; [g]Digestible energy was calculated on the basis of physiological fuel values 18.82, 14.64 and 35.55 kJ/g for protein, carbohydrate and fat, respectively (Jauncey 1982).

Table 2 Analyzed amino acid composition of the basal diet[a]

Amino acid	Basal diet (%)
EAAs	
Arginine	2.13
Histidine	0.73
Isoleucine	1.17
Leucine	0.73
Lysine	2.40
Methionine	1.35
Phenylalanine	2.07
Threonine	1.43
Tryptophan	0.51
Valine	1.02
NEAAs	
Cystine	0.82
Tyrosine	1.52
Alanine	1.87
Aspartic acid	1.17
Glutamic acid	2.36
Glycine	9.94
Proline	2.79
Serine	0.58

[a]Determined by Hitachi L-8800 Automatic Amino Acid Analyzer

Table 3 Growth, feed conversion, protein gain, leucine gain and RNA/DNA ratio of fingerling *C. catla* fed diets containing varying levels of leucine[a,b]

	Analyzed dietary leucine levels (%)					
	0.73 (L1)	0.97 (L2)	1.24 (L3)	1.46 (L4)	1.74 (L5)	1.97 (L6)
Average initial weight (g)	0.66±0.02[a]	0.65±0.03[a]	0.64±0.02[a]	0.64±0.08[a]	0.66±0.1[a]	0.64±0.05[a]
Average final weight (g)	3.07±0.05[e]	5.04±0.02[d]	6.05±0.06[c]	7.21±0.02[b]	8.11±0.07[a]	7.55±0.04[b]
Absolute weight gain (g/fish)	2.41±0.04[e]	4.39±0.02[d]	5.41±0.05[c]	6.57±0.02[b]	7.45±0.04[a]	6.91±0.06[ab]
Feed intake (g/fish)	10.71±0.08[a]	10.80±0.11[a]	10.79±0.06[a]	10.84±0.09[a]	11.24±0.05[a]	10.85±0.12[a]
Feed conversion ratio	4.43±0.04[a]	2.46±0.02[b]	1.99±0.01[c]	1.65±0.02[d]	1.51±0.05[e]	1.57±0.02[e]
Protein gain (g/fish)	0.29±0.01[e]	0.64±0.02[d]	0.87±0.01[c]	1.12±0.02[b]	1.31±0.04[a]	1.20±0.05[b]
Leucine gain (mg/fish)	23.95±0.72[f]	42.91±0.81[e]	61.59±1.21[d]	79.33±1.24[c]	85.33±1.39[a]	83.25±1.43[b]
RNA/DNA ratio	2.18±0.04[e]	3.14±0.05[d]	3.69±0.08[c]	4.11±0.04[b]	4.62±0.07[a]	4.56±0.05[a]

[a] Mean values of 3 replicates ± SEM. [b]Mean values sharing the same superscripts in the same row are insignificantly different (P>0.05).

Table 4 Carcass composition (wet basis) of fingerling *C. catla* fed diets containing varying levels of leucine[a,b]

	Analyzed dietary leucine levels (%)						
	Initial	0.73 (L1)	0.97 (L2)	1.24 (L3)	1.46 (L4)	1.74 (L5)	1.97 (L6)
Moisture (%)	78.94±0.54	79.42±0.52[a]	78.45±0.41[b]	77.2 3±0.4[c]	76.18± 0.31[d]	75.22± 0.42[e]	74.37± 0.58[f]
Protein (%)	12.48±0.13	12.24±0.06[e]	14.26±0.13[d]	15.71±0.07[c]	16.63±0.12[b]	17.16±0.08[a]	16.98±0.11[ab]
Fat (%)	3.48± 0.11	3.21± 0.02[f]	3.57± 0.04[e]	3.94± 0.03[d]	4.36± 0.02[c]	4.95± 0.05[b]	5.21± 0.08[a]
Ash (%)	2.51± 0.04	2.53±0.02[a]	2.56± 0.01[a]	2.54±0.02[a]	2.55± 0.03[a]	2.54± 0.02[a]	2.53± 0.04[a]

[a]Mean values of 3 replicates ± SEM. [b]Mean values sharing the same superscripts in the same row are insignificantly different (P>0.05).

Table 5 Hematological parameters of fingerling *C. catla* fed diets with varying levels of leucine[a,b]

	Analyzed dietary leucine levels (%)					
	0.73 (L1)	**0.97 (L2)**	**1.24 (L3)**	**1.46 (L4)**	**1.74 (L5)**	**1.97 (L6)**
Hemoglobin (g/dl)	3.73±0.02[e]	5.48±0.04[d]	7.52±0.06[c]	8.21±0.09[b]	9.28±0.11[a]	8.36±0.08[b]
Hematocrit (%)	12.21±0.61[e]	18.35±0.84[d]	25.86±0.68[c]	27.11±0.81[b]	30.26±0.92[a]	27.81±0.79[b]
Red blood corpuscles (10^6/mm^3)	1.99±0.03[e]	2.23±0.06[d]	2.31±0.06[c]	2.39±0.05[b]	2.68±0.02[a]	2.41±0.04[b]

[a]Mean value of 3 replicates±SEM. [b]Mean values with the same superscripts in a row are insignificantly different (P>0.05)

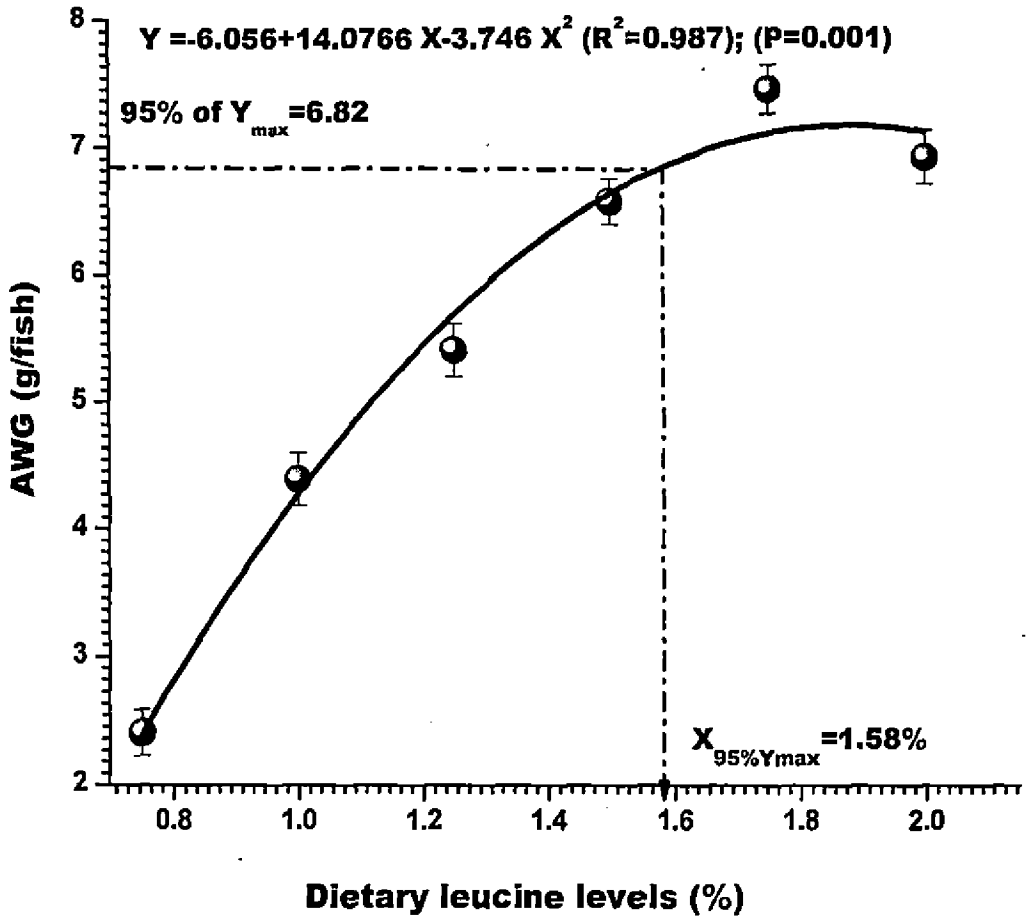

Fig. 1 Quadratic relationship of absolute weight gain to dietary leucine levels

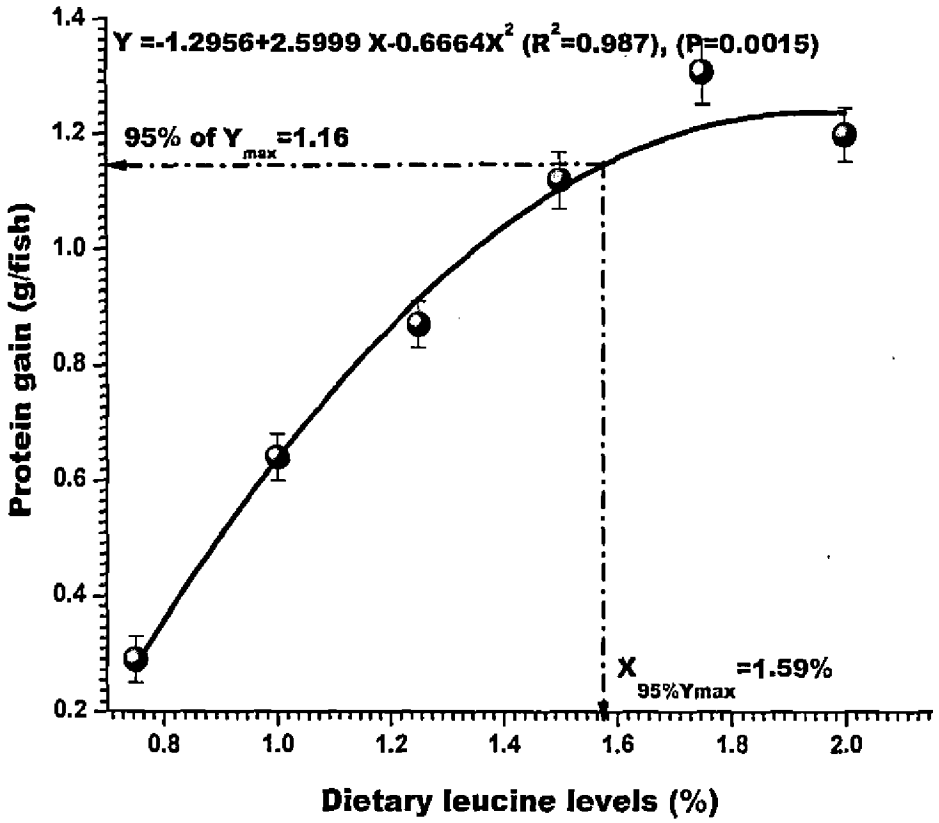

Fig. 2 Quadratic relationship of protein gain to dietary leucine levels

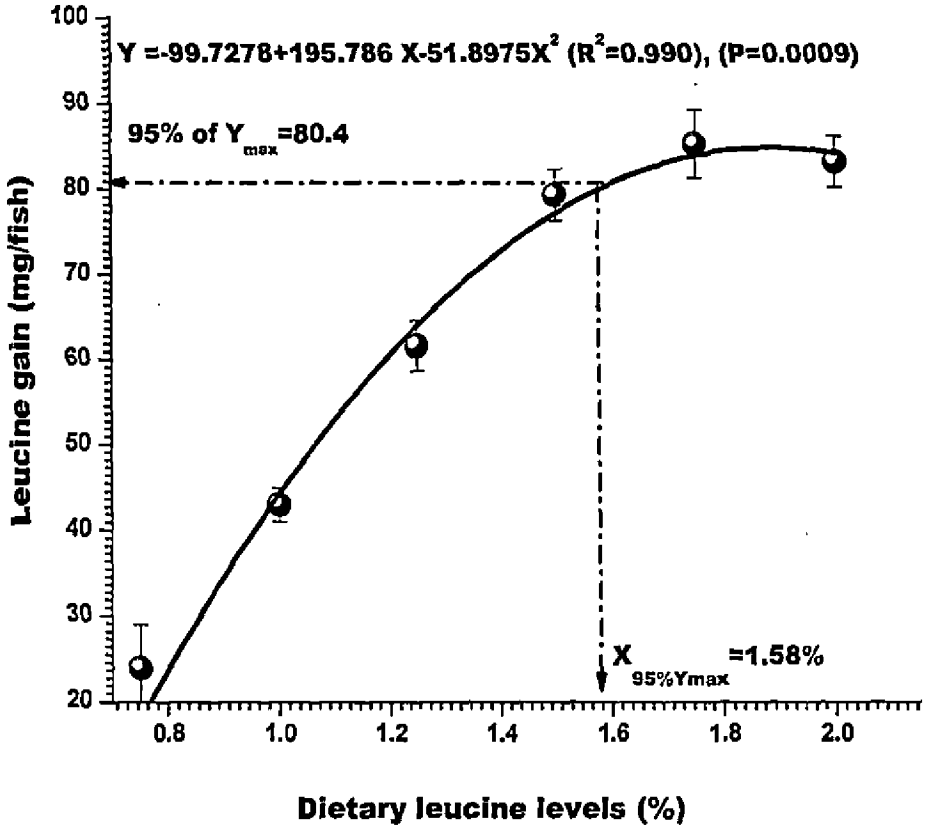

Fig. 3 Quadratic relationship of leucine gain to dietary leucine levels

CHAPTER 6

CHAPTER 6

DIETARY THREONINE REQUIREMENT OF INDIAN MAJOR CARP FINGERLING *CATLA CATLA* (HAMILTON) BASED ON GROWTH, PROTEIN RETENTION EFFICIENCY, THREONINE DEPOSITION, HEMATOLOGICAL PARAMETERS AND CARCASS COMPOSITION

INTRODUCTION

Lack of complete information on nutritional requirement is the most important factor hampering efforts in developing formula feeds for the intensive culture of Indian major carps. Any balanced formula for fish diet must include an energy source plus sufficient indispensable amino acids, essential fatty acids, phospholipids, specific vitamins and minerals to sustain life and promote growth (Halver et al. 1958). Amino acids, the building blocks of proteins, also play a pivotal role in intermediary metabolism (Engelen et al. 2001). The double function of amino acids as a catabolic fuel for energy metabolism and as an anabolic substrate for protein synthesis in aquaculture makes their supply crucial (Wright and Fyhn 2001). Provision of essential amino acid balanced feed improves the growth and health status of fish. The excess of essential amino acids negatively affect growth performance of fish. Thus, more specific knowledge about the requirements of the individual amino acid is important in formulating balanced feeds.

After lysine and methionine, threonine is usually one of the most limiting amino acids in practical ingredients (Ojano-Diranin and Waldroup 2002). Threonine participates in protein synthesis, and its catabolism generates many products important in metabolism (i.e., glycine, acetyl-CoA and pyruvate). It acts as a precursor of glycine and serine, is involved in immune responses, needed in gastrointestinal mucin production (Lemme 2003). Adequate inclusion of dietary threonine is essential to support optimum growth of aquaculture because it serves as an important component of body protein. Dietary threonine requirements have been worked out for mrigal, *Cirrhinus mrigala*; common carp, *Cyprinus carpio*; rohu, *Labeo rohita*; channel catfish, *Ictalurus punctatus;* stinging catfish, *Heteropneustes fossilis*, red drum, *Sciaenops ocellatus*; Nile tilapia, *Oreochromis*

niloticus; rainbow trout, *Oncorhynchus mykiss*; chum salmon, *O. keta*; pacific salmon, *Oncorhynchus spp.* and Atlantic Salmon, *Salmo salar* (NRC 2011).

Although threonine requirement of the fry stage of *C. catla* has been reported (Ravi and Devaraj 1991), information on the fingerling stage of this fish is completely lacking. Therefore, this study was carried out to determine the threonine requirement of fingerling *C. catla*.

Blood parameters are considered as patho-physiological indicator of the whole body and, therefore, important in diagnosing the structural and functional status of the fish under various stresses (Adhikari et al. 2004) and in determining the health status of the fish in response to the dietary supplements (Congleton and Wagner 2006; Mohammed and Sambo 2007). Moreover, hematological indices provide quite frequently and routinely accepted methods in aquaculture to evaluate the interactions between dietary levels of nutrients (Lim et al. 2000). Considering the significance of haematological indices in regulating the health condition of fish, these parameters have also been utilized in addition to the growth parameters for evaluating the threonine requirement of fingerling *C. catla.*

MATERIALS AND METHODS

Experimental diets

Six isonitrogenous (33% crude protein) and isocaloric (16.72 kJ/g gross energy) amino acid test diets using casein (fat-free), gelatin and crystalline L-amino acids with graded levels of threonine (0.75, 1.00, 1.25, 1.50, 1.75 and 2.00% dry diet) were formulated. The diets were designated as T1, T2, T3, T4, T5 and T6. The levels of threonine in the amino acid test diets were fixed on the basis of information available on other carps (NRC 2011). Crystalline L-amino acids, excluding the test amino acid threonine, were used to simulate the amino acid profile of the experimental diets to that of 33% whole chicken egg protein. The composition of the basal diet is given in Table 1. The amount of threonine contributed by the casein and gelatin in basal diet was 0.74%. The amount of threonine was increased at the expense of glycine, on protein to protein basis to attain the

intended concentrations of dietary threonine. The analyzed threonine contents of the experimental diets were found to be at 0.74, 0.96, 1.21, 1.48, 1.72 and 1.93% dry diet, respectively. The analyzed amino acid composition of the basal diet is presented in Table 2. Method of preparation of experimental diets has been discussed under the General Methodology section (pages 9-10).

Experimental design and feeding trial

Source of the fish, their acclimation and details of the general experimental design has already been discussed under the General Methodology section (page 8).

Fingerling *C. catla* (3.35±0.11 cm; 0.59±0.06 g) were taken from the above acclimated fish lot and stocked in 70 L circular polyvinyl troughs (water volume 55 L) fitted with a continuous water flow-through system (1-1.5 L/min) at the rate of 25 fish per trough in triplicate groups for each dietary treatment. Fish were fed test diets in the form of dry crumbles (500 µm) to apparent satiation thrice daily at 08:00, 12:30 and 17:30h. Initial and weekly weights were recorded on a top-loading balance (Precisa 120A; 0.1 mg sensitivity, Oerlikon AG, Zurich, Switzerland) after anaesthetizing the fish with tricaine methane sulfonate (MS-222; 100 µg/ml). Fish were deprived of feed on the day they were weighed. The feeding trial lasted for 12 weeks. Faecal matter was siphoned before every feeding. Water quality indices were monitored daily during the feeding trial and were recorded following standard methods (APHA 1992). The range of water temperature, dissolved oxygen, free carbon dioxide, pH, total ammonia nitrogen, nitrites and total alkalinity, based on daily measurements, was 26.2-27.5°C, 6.8-7.3 mg/L, 7.2-9.3 mg/L, 7.5-7.8, 0.27-0.33 mg/L, 0.05-0.09 mg/L and 77.4-80.1 mg/L, respectively.

Sample collection and chemical analyses

At the beginning of the feeding trial, 60 fish were randomly sampled, killed after anaesthetizing with MS-222 (100 µg/ml) and pooled together. Six subsamples of a pooled sample were analyzed for initial fish carcass composition. At the end of the experiment, 20 fishes from each replicate of dietary treatments were randomly collected, sacrificed with an overdose of MS-222 and pooled separately. Three subsamples of the pooled

samples were analyzed for final carcass composition. Proximate composition of casein, gelatin, experimental diets, and initial and final carcass was estimated using standard methods (pages 10-11). Gross energy content was determined on a Gallenkamp Ballistic Bomb Calorimeter as per the method described on page 12. Amino acid analysis of casein, gelatin, experimental diets, initial and final fish carcass was done using an automatic amino acid analyzer as detailed earlier (page 12).

Determination of RNA and DNA

RNA and DNA were determined by the method of Schneider (1957) as detailed earlier on page 13.

Hematological analyses

At the termination of the feeding trial, blood samples were collected in heparinized vials through cardiac puncture of the fish. The blood of five fish from each replicate of the treatment group was pooled to obtain enough samples for hematological analysis. Analysis of red blood cell counts, hemoglobin and hematocrit value were done as per the method detailed on page 88.

Evaluation of growth parameters

Calculation of various growth parameters was made according to the standard definitions as described under the General Methodology section (pages 13-14).

Statistical analyses

Statistical analyses of growth data were done using procedures as detailed earlier (page 14).

RESULTS

Growth performance

Absolute weight gain (AWG), feed conversion ratio (FCR), protein retention efficiency

(PRE%) and threonine deposition (TD) were significantly affected by the varying concentrations of dietary threonine (Table 3). Fish fed diet with 1.48% threonine (T4) reflected maximum AWG (7.99 g/fish), PRE (33.66%), TD (0.64) and best FCR (1.48). Quadratic regression analysis at 95% maximum response of AWG (Fig. 1), PRE, TD (Fig. 2) and minimum response of FCR against varying levels of dietary threonine exhibited the requirement between 1.35-1.48% dry diet, corresponding to 4.09-4.48% dietary protein. The equations used to fit the data are as follows:

$$Y=-6.40325X^2+21.04938X-9.22102\ (R^2=0.997)\ X_{95\%ymax}=1.39\%\ (AWG)$$

$$Y=-26.627X^2+92.363X-46.5305\ (R^2=0.989)\ X_{95\%ymax}=1.48\%\ (PRE)$$

$$Y=-0.52768X^2+1.66346X-0.52768\ (R^2=0.978)\ X_{95\%ymax}=1.35\%\ (TD)$$

$$Y=3.74904X^2-11.9177X+10.73056\ (R^2=0.945)\ X_{95\%ymin}=1.46\%\ (FCR)$$

All the diets were well accepted by the experimental fish and no significant differences in feed intake were found among all the treatments (Table 3).

Carcass composition

Changes in the carcass composition of fingerling *C. catla* fed diets with different levels of threonine are shown in Table 4. Increase in the dietary concentrations of threonine produced significant increase in carcass protein up to 1.48% (T4). The relationship of carcass protein to dietary threonine was expressed by the quadratic equation ($Y=-3.5771X^2+13.581X-3.277$; $R^2=0.953$). On solving this equation, the 95% maximum response is obtained at 1.42% dietary threonine, corresponding to 4.30% dietary protein. Lowest moisture content was recorded in fish fed 0.74% threonine diet (T1) whereas highest moisture content was noted in fingerling *C. catla* fed 1.93% threonine diet (T6). Carcass fat was found to decrease linearly with the increase in dietary threonine concentrations up to 1.48% (T4) and remained almost constant beyond above level. No significant differences in carcass ash content was recorded in fish fed all the diets. Survival was not affected by the varying levels of dietary threonine and remained 100% in all the groups.

Nucleic acid indices

The RNA/DNA ratio was significantly affected by dietary threonine content (Table 4). The RNA/DNA ratio significantly increased with the increasing levels of dietary threonine up to 1.48% (T4). No significant differences in RNA/DNA ratio were noted in fish fed diet T5. However, significant decline in RNA/DNA ratio was noted in fish fed T6 diet. Quadratic regression analysis of RNA/DNA ratio against dietary threonine predicted the 95% maximum response of RNA/DNA ratio to be at 1.44% threonine of the diet, corresponding to 4.36% dietary protein. The relationship being;

$$Y=-3.65479\ X^2+12.439X-5.52458\ (R^2=0.967)\ X_{95\%ymax}=1.44\%$$

Somatic indices

Dietary threonine levels had the significant impact ($P<0.05$) on somatic indices (Table 4). The pattern of HSI was found to be negatively correlated with the increasing concentrations of dietary threonine up to 1.48% (T4) followed by insignificant change in fish fed diets T5 and T6. The CF showed positive and significant ($P<0.05$) trend with the increase of dietary threonine up to 1.48% beyond which no further increase ($P>0.05$) in CF was noted. Contrary to this, VSI was found to decrease with the increasing concentrations of dietary threonine up to the above level of dietary threonine. Further increase in dietary threonine did not show any significant change in VSI.

Effect of threonine on hematological parameters

Data pertaining to hematological parameters in response to increasing levels of dietary threonine are provided in table 5. Hemoglobin (g/dl), hematocrit (%) and RBCs ($10^6/mm^3$) significantly ($P<0.05$) increased with the increase in dietary threonine up to 1.48% (T4). These parameters remained insignificantly ($P>0.05$) different in fish fed diet T5 and, thereafter, declined significantly. Quadratic regression analysis of hemoglobin ($Y=-5.7664X^2+19.299\ X-7.1334$; $R^2=0.993$; $X_{95\%ymax}=1.41\%$), hematocrit ($Y=-17.934X^2+59.3796X+21.308$; $R^2=0.966$ $X_{95\%ymax}=1.39\%$) and RBCs ($Y=-1.0386X^2+3.5334\ X-1.03861$; $R^2=0.944$; $X_{95\%ymax}=1.36\%$) against dietary threonine

concentrations reflected the threonine requirement between 1.36-1.41% dry diet.

Threonine requirement

Based on quadratic regression analysis of absolute weight gain, feed conversion ratio, protein retention efficiency%, threonine deposition, carcass protein, RNA/DNA ratio, hemoglobin, hematocrit (%) and RBCs at 95% of maximum and minimum response, dietary threonine requirement of fingerling *C. catla* was found to range between 1.35-1.48% dry diet, corresponding to 4.09-4.48% dietary protein.

DISCUSSION

The threonine requirement of different fish species varies between 1.8 to 5.3% of the dietary protein (NRC 2011). The threonine requirement of this fish species based on quadratic regression analysis of above parameters was found to range between 1.35-1.48% dry diet, corresponding to 4.09-4.48% dietary protein. This requirement (4.09-4.48% dietary protein) is higher than the requirements reported for Japanese flounder *Paralichthys olivaceus* 3.1% (Alam et al. 2003); gilthead sea bream *Sparus aurata* 2.8% (Kaushik 1998); turbot *Scophthalmus maximus* 2.9% (Kaushik 1998); rohu *L. rohita* 3.8-4.2% (Abidi and Khan 2008); common carp *C. carpio* 3.9% (NRC 2011) and comparable to the requirement of rohu *L. rohita* 4.3% (Murthy and Varghese 1996b); mrigal *C. mrigala* 4.5% (Ahmed et al. 2004) of the dietary protein. The reported variation in amino acid requirements may be the result of laboratory variances: different experimental diets, feeding levels, frequency, size and age of fish, genetic differences, rearing conditions, environmental conditions such as water quality and water flow rate (Chiu et al. 1988; Coloso et al. 1999; Simmons et al. 1999; De Silva et al. 2000). The real disparity observed between the threonine requirements of the fish may also be due to the differences in the methodologies used such as the nature of the dietary protein sources in the test diets, the reference protein, the amino acid pattern being mimicked and the culture conditions (Benakappa and Varghese 2002). Different statistical models may also be responsible for the variation in the threonine requirements of the fish.

Dietary threonine requirement of fingerling *C. catla* (4.09-4.48% dietary protein)

in this study is lower than the requirement (4.9% dietary protein) reported by Ravi and Devaraj (1991) for fry stage of this fish. Lower threonine requirement obtained for fingerling *C. catla* in this study compared to higher threonine requirement reported by Ravi and Devaraj (1991) on fry may be due to differences in fish size as larger fish may have lower requirement (Sugiura et al. 2000). A feeding frequency of three times a day *ad lib* adopted in this study compared to feeding frequency of two times a day at a fixed ration level by Ravi and Devaraj (1991) may be the reason for the differences in the threonine requirement of *C. catla*. Different dietary protein levels adopted in this study (33% dry diet) and that used (40% dry diet) by Ravi and Devaraj (1991) may also be responsible for the variation in the threonine requirements of this fish. Coating of crystalline amino acids in this study may also lead to different threonine requirement. It has been reported that coating of crystalline amino acids in diet improves the utilization of crystalline amino acids by minimizing the leaching loss and alleviating the absorption rate of free amino acids to form a more balanced amino acid pool beneficial for protein synthesis (Murai et al. 1982). Ravi and Devaraj (1991) have used uncoated crystalline amino acids which were probably not properly utilized leading to higher estimate of threonine requirement. Whereas in this study crystalline amino acids were coated with carboxymethyl cellulose followed by casein and gelatin which regulate the absorption rate of amino acids leading to optimum estimate of threonine requirement. Moreover, requirement reported by Ravi and Devaraj (1991) is based on weight gain only which may be due to the accumulation of moisture content and, therefore, is not considered as a reliable parameter of growth. However, in this study, the requirement is recommended not on the basis of weight gain only but also on the basis of several sensitive and reliable parameters such as threonine deposition, carcass protein, hemoglobin, hematocrit and RBCs.

Both the broken-line and quadratic regression models have generally been used to quantify the nutrient requirements in aquatic species (Zeitoun et al. 1976). It has been reported that broken-line regression underestimates the requirement compared with quadratic regression analysis (Morris 1989; Baker et al. 2002). Hence, a quadratic model was adopted in this study to work out the threonine requirement of fingerling *C. catla*

which exhibited high R^2 estimates for AWG (0.997), PRE (0.988), TD (0.978), FCR (0.965), CP (0.953), RNA/DNA ratio (0.973), Hb (0.987); Ht (0.937), RBCs (0.949). The high values of coefficient of determination obtained in this study indicate the appropriateness of this model in estimating the threonine requirement.

The deficiency of certain essential amino acids has been shown to affect growth and feed conversion negatively in fish (Fauconneau et al. 1992; Benakappa and Varghese 2002; Khan and Abidi 2011a; Farhat and Khan 2013a) followed by increase nitrogen losses (Aragao et al. 2004; 2007). In this study, reduced gain in weight of fingerling *C. catla* fed diets containing sub-optimum levels of dietary threonine (T1-T3) may be due to amino acid imbalance in the body induced by the inadequate levels of threonine. However, the weight gain was found to maximum at 1.48% threonine diet (T4). No significant gain in weight of fish fed dietary threonine at 1.72% (T5) was recorded. However, decreased growth in fish fed diet T6 indicate that the threonine at high concentration was being catabolized and less was utilized for growth. Excess threonine may also lead to extra energy expenditure toward deamination and elimination of amino acids that have adverse effects on growth. Similar decline in growth in fish fed higher concentrations of dietary threonine has also been reported by Murthy and Varghese (1996b); Benakappa and Varghese (2002); Ahmed et al. (2004) and Abidi and Khan (2008).

Aquaculture feeds are formulated to maximize nutrient retention and minimize nutrient loss. This strategy is driven by both economic and environmental considerations (Pirozzi et al. 2010). Nutrients retention efficiencies have been shown to be influenced by the dietary concentrations of threonine. Dietary threonine is absorbed in the small intestine and used by the peripheral tissues mainly for protein deposition associated with growth and protein mass maintenance (Hamard et al. 2009). In this study, protein retention and threonine deposition in fingerling *C. catla* significantly improved with the increasing concentrations of threonine up to 1.48% of the dry diet (T4). The RNA/DNA ratio is considered as an extremely sensitive biochemical indicator of the physiological and nutritional state of aquatic organisms in natural environment (Mustafa 1977; 1979; 1983; Mustafa and Jafri 1977; Mustafa and Mittal 1982; Mustafa and Zofair 1985; Bulow

1987; Mustafa et al. 1991; Buckley et al. 1999; Chicharo and Chicharo 2008). In this study, RNA/DNA ratio showed a positive trend with the increasing concentrations of threonine up to 1.48% of the dry diet (T4). This significant improvement in RNA/DNA ratio and protein retention up to the above level of dietary threonine suggests proper utilization of threonine for body protein synthesis.

Fish hematology is gaining increasing importance in fish culture because of its importance in monitoring the health status of fish (Hrubec et al. 2000). Nutritional status of the fish is one of the most important variables altering the blood values (Congleton and Wagner 2006; Khan and Abidi 2011a; Farhat and Khan 2013a). In the present study, the hemoglobin (Hb), RBCs and hematocrit (Ht%) values were affected by increasing threonine concentrations and were found to be significantly improved with the increase in threonine up to 1.48% of the diet (T4). Maximum Hb, RBCs and hematocrit values obtained in fish fed diet at above level of threonine may be due to adequate synthesis of protein required for optimum growth and blood parameters.

Condition factor and hepatosomatic index are considered as indicators of fish health and used as an important tool in several amino acid requirements studies (Coloso et al. 2004; Marcouli et al. 2006; Espe et al. 2008; Farhat and Khan 2013b). A decrease in condition factor, the HSI, or both is considered a reflection of depletion in energy reserves (Goede and Barton 1990) because these indices are positively related to total muscle and liver energy content (Lambert and Dutil 1997). In this study, significant differences in CF were observed among fish fed dietary threonine ranging between 0.87-1.46%, and the best CF occurred in the group fed diet containing 1.48% threonine (T4) indicating that this group contain more energy reserves such as protein and fat, and thus are in better condition than those fed T1, T2 and T3 diets having lower condition factor. In the present study, HSI showed decreasing trend with the increasing concentrations of dietary threonine up to 1.48% (T4). The higher values of HSI at lower levels of dietary threonine (T1-T3) may be due to the conversion of unutilized amino acids to glucose under gluconeogenesis and stored in liver as glycogen rather than body protein synthesis. Contrary to this study, an insignificant change in HSI with the increase of dietary threonine has been reported by Silva (2006).

Carcass composition was significantly ($P<0.05$) affected by the varying levels of dietary threonine. Carcass protein was found to improve with the increasing concentrations of dietary threonine up to 1.48% (T4) beyond which (T5-T6) remained almost constant. Carcass fat negatively affects the quality of the final product by degrading the fish fillet through lipid oxidation. A reduction in carcass fat is often seen with better amino acid balance, but usually this is also accompanied by less amino acid catabolism and higher body protein synthesis (Grisdale-Helland et al. 2013). Hence, reduction in carcass fat may improve the carcass quality which is desirable in cultured fish. In this study, threonine deficient diets (T1-T3) resulted in high fat content than those fed 1.48 (T4), 1.72 (T5) and 1.93% dietary threonine (T6). Higher carcass fat recorded at sub-optimum levels of dietary threonine (T1-T3) in the present study may be attributable to the fact that threonine helps in the breaking down of fat during the metabolizing process (Rosa 2012). The lipolytic effect of threonine at sub-optimum levels has also been successfully established in rats (Singal et al. 1953).

Based on quadratic regression analysis of absolute weight gain, feed conversion ratio, protein retention efficiency, threonine deposition, carcass protein, RNA/DNA ratio, hemoglobin, hematocrit and RBCs, at 95% of maximum and minimum response, dietary threonine requirement of fingerling *C. catla* was found to range between 1.35-1.48% dry diet. Hence, inclusion of threonine in the range of 1.35-1.48% dry diet, corresponding to 4.09-4.48% dietary protein is optimum in formulating threonine-balanced feeds for this fish.

SUMMARY

A 12-week feeding trial was conducted to determine the dietary threonine requirement of fingerling Indian major carp, *Catla catla* (3.35±0.11 cm; 0.59±0.06 g). Six casein-gelatin based (33% crude protein; 16.72 kJ/g gross energy) amino acid test diets with graded levels of threonine (0.75, 1.00, 1.25, 1.50, 1.75, 2.00% dry diet) were fed to satiation to triplicate groups of fish. Analyzed threonine concentrations were 0.74, 0.96, 1.21, 1.48, 1.72 and 1.93% dry diet. Absolute weight gain (g/fish), feed conversion ratio, protein retention efficiency%, threonine deposition, RNA/DNA ratio and carcass protein

significantly improved with the increase in dietary threonine and peaked at 1.48% of the dry diet. Hematological indices were also found to be best in fish fed at 1.48% threonine diet. Quadratic regression analysis of absolute weight gain, feed conversion ratio, protein retention efficiency%, threonine deposition, RNA/DNA ratio, carcass protein, hemoglobin (g/dl), hematocrit (%) and RBCs (10^6/mm^3) at 95% of maximum and minimum response exhibited the threonine requirement of fingerling *C. catla* between 1.35-1.48% dry diet, corresponding to 4.09-4.48% dietary protein. Present finding would be useful in formulating threonine balanced feeds for the intensive culture of *C. catla*.

Table 1 Composition of the basal diet for fingerling *C. catla* (initial body weight=0.59±0.06 g/fish)

Ingredients	% dry diet
Casein[a] (fat-free)	18
Gelatin[b]	6
Dextrin	33.17
Amino acid mixture[c]	14.04
Corn oil	5
Cod liver oil	2
Mineral mix[d,f]	4
Vitamin mix[e,f]	3
α- Cellulose	4.79
Carboxymethyl cellulose	10
Total	100
Analyzed crude protein	32.63
Digestible energy[g] (kJ/g, dry diet)	13.21
Calculated gross energy (kJ/g, dry diet)	16.72

[a]Crude Protein (76%); [b]Crude Protein (96%); [c]Amino acid mixture (g/100 g dry diet) arginine 0.973, histidine 0.174, isoleucine 1.567, leucine 1.124, lysine 0.633, methionine 0.718 cystine 0.712 phenylalanine 1.072, tyrosine 0.560, tryptophan 0.356, valine 1.097, alanine 0.748 glycine 4.265 (Loba Chemie, India), proline 0.079; Loba Chemie, Mumbai, India. [d]Mineral mixture (g/100 g of mineral mix) calcium biphosphate 13.57; calcium lactate 32.69; ferric citrate 02.97; magnesium sulphate 13.20; potassium phosphate (dibasic) 23.98; sodium biphosphate 08.72; sodium chloride 04.35; aluminium chloride. $6H_2O$ 0.0154; potassium iodide 0.015; cuprous chloride 0.010; manganous sulphate. H_2O 0.080; cobalt chloride. $6H_2O$ 0.100; zinc sulphate. $7H_2O$ 0.40; [e]Vitamin mixture (g/100 g dry diet) choline chloride 0.500; inositol 0.200; ascorbic acid 0.100; niacin 0.075; calcium pantothenate 0.05; riboflavin 0.02; menadione 0.004; pyridoxine hydrochloride 0.005; thiamin hydrochloride 0.005; folic acid 0.0015; biotin 0.0005; alpha-tocopherol 0.04; vitamin B_{12} 0.00001; 2.0 g α-cellulose; Loba Chemie, India; [f]Halver (2002); [g]Digestible energy was calculated on the basis of physiological fuel values 18.83, 14.64 and 35.56 kJ/g for protein, carbohydrate and fat, respectively (Jauncey 1982).

Table 2 Analyzed amino acid composition of the basal diet for fingerling *C. catla* (initial body weight=0.59±0.06 g/fish)[a]

Amino acid	Casein	Gelatin	Added crystalline L-amino acids	Basal diet (g/100g)
EAAs				
Arginine	0.65	0.49	0.97	2.15
Histidine	0.47	0.05	0.17	0.70
Isoleucine	1.01	0.06	1.57	2.63
Leucine	1.76	0.16	1.12	3.02
Lysine	1.48	0.24	0.66	1.78
Methionine	0.61	0.03	0.72	1.32
Phenylalanine	0.90	0.11	1.07	2.09
Threonine	0.65	0.09	0.01	0.74
Tryptophan	0.14	0	0.36	0.51
Valine	1.22	0.09	1.10	2.46

[a]Determined by Hitachi L-8800 Automatic Amino Acid Analyzer

Table 3 Growth performance of fingerling *C. catla* (initial body weight=0.59±0.06 g/fish) fed diets with varying levels of threonine[a,b]

	Analyzed dietary threonine levels (%)					
	0.74	**0.96**	**1.21**	**1.48**	**1.72**	**1.93**
Average initial weight (g)	0.59±0.04[a]	0.60±0.05[a]	0.60±0.04[a]	0.60±0.02[a]	0.59±0.07[a]	0.60±0.05[a]
Average final weight (g)	3.40±0.08[d]	5.79±0.09d[c]	7.44±0.11[b]	8.59±0.12[a]	8.40±0.11[ab]	8.29±0.14[b]
Absolute weight gain (g/fish)	2.81±0.07[d]	5.19±0.05[c]	6.84±0.08[b]	7.99±0.06[a]	7.81±0.09[ab]	7.69±0.10[b]
Feed intake (g/fish)	11.83±0.10[a]	12.14±0.12[a]	12.17±0.11[a]	11.83±0.14[a]	11.64±0.18[a]	11.76±0.13[a]
Feed conversion ratio	4.21±0.05[a]	2.34±0.06[b]	1.78±0.02[c]	1.48±0.03[e]	1.49±0.04[e]	1.53±0.05[d]
Protein retention efficiency%	7.88±0.11[e]	16.92±0.16[d]	25.03±0.27[c]	33.66±0.41[a]	33.32±0.35[a]	32.22±0.37[b]
Threonine deposition	0.26±0.02[e]	0.39±0.04[d]	0.53±0.02[c]	0.64±0.02[a]	0.61±0.03[a]	0.54±0.03[b]

[a]Mean values of 3 replicates ± SEM; [b]Mean values sharing the same superscript are not significantly different (P>0.05).

Table 4 Carcass composition (wet basis), RNA/DNA ratio and somatic indices of fingerling *C. catla* (initial body weight=0.59±0.06 g/fish) fed diets with varying levels of threonine[a, b]

	Analyzed dietary threonine levels (%)						
	Initial	0.74	0.96	1.21	1.48	1.72	1.93
Moisture (%)	79.12±1.28	74.23±1.52[b]	75.12±1.41[ab]	76.25±1.46[ab]	77.31±1.59[ab]	78.21±1.51[ab]	79.11± 1.42[a]
Protein (%)	12.23±0.14	11.21±0.31[d]	12.98±0.62[c]	14.51±0.58[b]	16.15±0.53[a]	16.11±0.49[a]	15.97±0.52[a]
Fat (%)	2.64±0.05	4.94± 0.06[a]	4.18± 0.08[b]	3.12± 0.07[c]	2.21±0.03[d]	2.23± 0.06[d]	2.24± 0.05[d]
Ash (%)	2.11±0.08	2.21±0.05[a]	2.19± 0.06[a]	2.21±0.03[a]	2.19±0.06[a]	2.19± 0.04[a]	2.21± 0.02[a]
RNA/DNA ratio	1.74±0.04	1.81±0.04[e]	2.97±0.09[d]	3.82±0.04[c]	5.21±0.05[a]	5.19±0.08[a]	4.71±0.07[b]
HSI%[c]	-	0.97±0.07[a]	0.89±0.06[b]	0.82±0.05[c]	0.77±0.04[cd]	0.76±0.02[d]	0.74±0.02[d]
CF[d]	-	0.87±0.03[d]	1.21±0.07[c]	1.32±0.05[b]	1.46±0.04[a]	1.46±0.05[a]	1.45±0.04[a]
VSI%[e]	-	5.41±0.14[a]	4.85±0.13[b]	4.48±0.11[c]	4.02±0.14[d]	4.04±0.16[d]	4.06±0.12[d]

[a]Mean values of 3 replicates ± SEM; [b]Mean values sharing the same superscript are not significantly different (P>0.05)

[c]Hepatosomatic index

[d]Condition factor

[e]Viscerosomatic index

Table 5 Hematological parameters of fingerling *C. catla* fed diets with varying levels of dietary threonine[a,b]

	Analyzed dietary threonine levels (%)					
	0.75	**1.00**	**1.25**	**1.50**	**1.75**	**2.00**
Hemoglobin (g/dl)	4.12±0.04[e]	5.87±0.06[d]	7.71±0.09[c]	9.05±0.06[a]	8.92±0.12[ab]	8.61±0.10[b]
Hematocrit (%)	13.87±0.84[e]	17.26±0.92[d]	24.43±0.71[c]	28.38±0.65[a]	27.74±0.87[a]	26.13±0.81[b]
Red blood corpuscles (10^6/mm^3)	1.94±0.02[e]	2.21±0.04[d]	2.47±0.06[c]	2.91±0.05[a]	2.89±0.06[a]	2.72±0.07[b]

[a]Mean value of 3 replicates±SEM. [b]Mean values with the same superscripts in a row are insignificantly different (P>0.05)

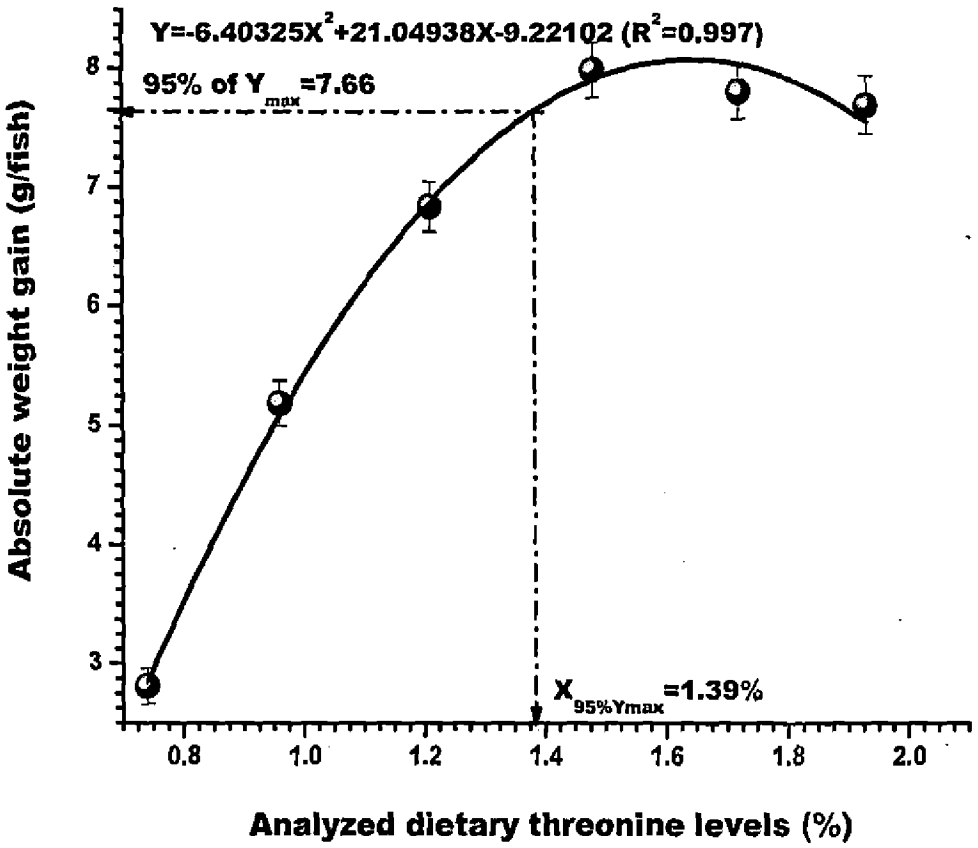

Fig 1. Quadratic relationship of absolute weight gain to dietary threonine concentrations in fingerling *C. catla* (initial body weight=0.59±0.06 g/fish) for 12 weeks

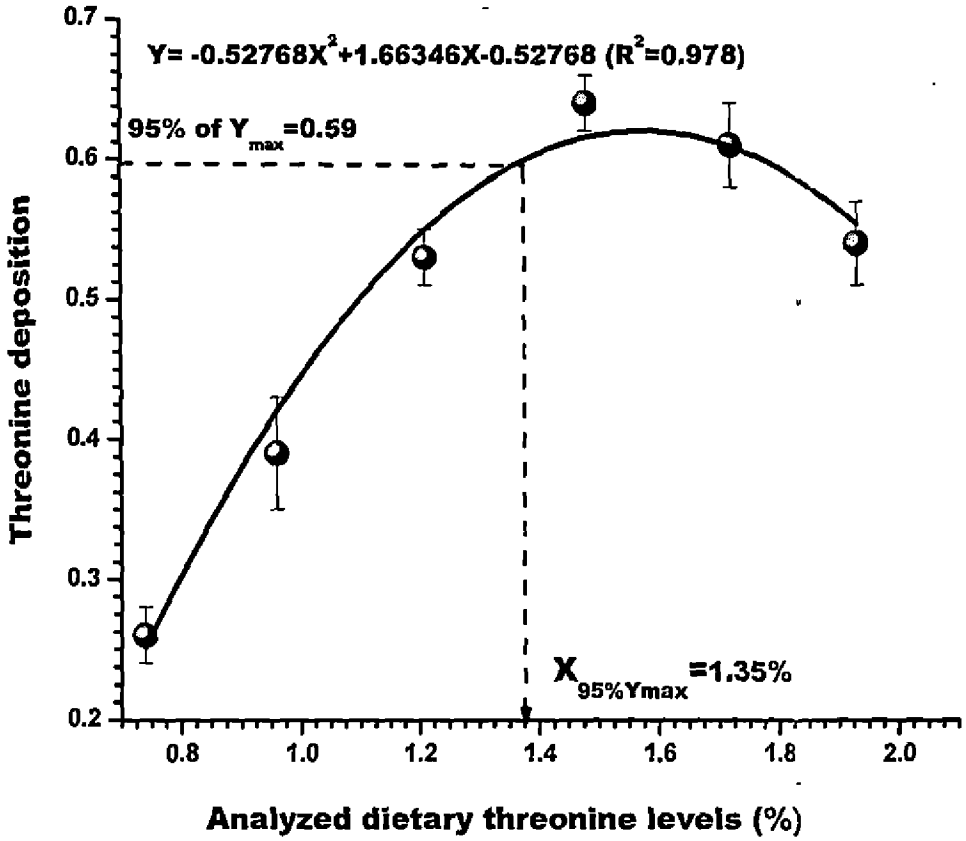

Fig 2. Quadratic relationship of threonine deposition to dietary threonine concentrations in fingerling *C. catla* (initial body weight=0.59±0.06 g/fish) for 12 weeks

THESIS

CHAPTER 7

CHAPTER 7

DIETARY TRYPTOPHAN REQUIREMENT OF FINGERLING *CATLA CATLA* (HAMILTON) BASED ON GROWTH, PROTEIN GAIN, RNA/DNA RATIO, HEMATOLOGICAL PARAMETERS AND CARCASS COMPOSITION

INTRODUCTION

Dietary amino acids, due to its impact on fish growth and cost, have received priority in fish nutrition studies. Fish at early life stages show rapid growth and hence delivery of amino acids during this period is critical for body protein synthesis and energy (Terjesen et al. 2006). Tryptophan is an indispensable amino acid required for a wide variety of metabolic activities. Because its concentration in organisms is lowest among the all amino acids, it can easily play a rate-limiting role in protein synthesis (Emadi et al. 2010). Moreover, it serves as the precursor of serotonin, melatonin, tryptamine, NAD, and NADP, as well as meeting the majority of the requirement for nicotinic acid (Sainio et al. 1996). Feeding tryptophan-supplemented diet resulted in the inhibition of endogenously derived aggressive behavior (Hseu et al. 2003). Tryptophan is known to be stress suppressor in some species (Lepage et al. 2003; Tejpal et al. 2009). It has also been reported that optimized inclusion of tryptophan in diet improved the hematological parameters (Farhat and Khan 2012b; Ahmed 2012a). Dietary tryptophan requirement has been worked out for a number of finfish species such as rohu, *Labeo rohita*; mrigal, *Cirrhinus mrigala*; common carp, *Cyprinus carpio*; tilapia, *Oreochromis spp.*; channel catfish, *Ictalurus punctatus;* rainbow trout, *Oncorhynchus mykiss*; Atlantic salmon, *salmo salar*; pacific salmon, *O. spp.* (NRC 2011) and stinging catfish *Heteropneustes fossilis* (Farhat and Khan 2012b; Ahmed 2012a).

One of the prerequisites for developing quality feeds for *C. catla* is the understanding of its nutritional requirements particularly in terms of essential amino acid needs as the sustainability of aquaculture depends on the development of amino acid balanced feed. Therefore, information on tryptophan requirement is important for the formulation of tryptophan-balanced feeds for intensive culture of this fish species.

Although information on dietary tryptophan requirement of fry *C. catla* exists (Ravi and Devaraj 1991), no published information on dietary tryptophan requirement of fingerling stage of this fish is available. Therefore, the present work was performed to establish the tryptophan requirement of fingerling *C. catla*.

MATERIALS AND METHODS

Experimental diets

Six isonitrogenous (33% crude protein) and isocaloric (16.72 kJ/g gross energy) amino acid test diets using casein (vitamin and fat-free), gelatin and crystalline L-amino acids with graded levels of tryptophan (0.10, 0.15, 0.20, 0.25, 0.30 and 0.35% dry diet) were formulated. Analyzed amino acid composition of diets reflected the L-tryptophan content to be 0.10, 0.14, 0.19, 0.23, 0.28 and 0.34% of the dry diet. The diets were designated as T1, T2, T3, T4, T5 and T6. The levels of tryptophan in the amino acid test diets were fixed on the basis of information available on other carps (NRC 2011). Tryptophan gets converted to niacin (Nishizuka and Hayaishi 1963; Goldsmith et al. 1961) and hence the requirement may be affected by the availability of dietary niacin. To rule out the possibility of conversion of tryptophan to niacin, required amount of niacin in the test diets was fixed on the basis of information available on other two Indian major carps namely rohu and mrigal (Ahmed 2011). Crystalline L-amino acids excluding the tryptophan were used to simulate the amino acid profile of the experimental diets to that of 33% whole chicken egg protein. The composition of the basal diet is presented in Table 1. The basal diet used to quantify the tryptophan requirement of fingerling *C. catla* contained 0.1% dietary tryptophan from casein. The amount of tryptophan was increased at the expense of glycine, on protein to protein basis to attain the desired concentrations of dietary tryptophan. The analyzed amino acid composition of the basal diet is presented in Table 2. Method of preparation of experimental diets has been discussed under General Methodology section (pages 9-10).

Experimental design and feeding trial

Source of the fish, their acclimation and details of the general experimental design has

already been discussed under the General Methodology section (page 8).

Fingerling *C. catla* (3.45±0.24 cm; 0.60±0.13 g) were taken from the above acclimated fish lot and stocked in 70 L circular polyvinyl troughs (water volume 55 L) fitted with a continuous water flow-through (1-1.5 L/min) system in triplicate groups at the rate of 25 fish per trough for each dietary treatment. Fish were fed test diets in the form of dry crumbles (500 µm) to apparent satiation thrice daily at 08:00, 12:30 and 17:30h. Initial and weekly weights were recorded on a top-loading balance (Precisa 120A; 0.1 mg sensitivity, Oerlikon AG, Zurich, Switzerland) after anaesthetizing the fish with tricaine methane sulfonate (MS-222; 100 µg/ml). Fish were deprived of feed on the day they were weighed. The feeding trial lasted for 12 weeks. Faecal matter was siphoned before every feeding. Water quality indices were monitored daily during the feeding trial and recorded following standard methods (APHA 1992). The range of water temperature, dissolved oxygen, free carbon dioxide, pH, and total alkalinity based on daily measurements, was 27.3-28.1°C, 6.9-7.6 mg/L, 5.5-9.3 mg/L, 7.3-7.6 and 65.4-82.6 mg/L, respectively.

Hematological analyses

At the termination of the feeding trial, blood samples were collected in heparinized vials through cardiac puncture of the fish. The blood of five fish from each replicate of the treatment group was pooled to obtain enough samples for hematological analysis. Analysis of red blood cell counts, hemoglobin and hematocrit value were done as per the method detailed on page 88.

Chemical analyses

Proximate composition of casein, gelatin, experimental diets, and initial and final carcass was estimated using standard methods as detailed earlier on pages 10-11. Gross energy content was determined on a Gallenkamp Ballistic Bomb Calorimeter as per the method described on page 12. Amino acid analysis of casein, gelatin, experimental diets, initial and final fish carcass was done using an automatic amino acid analyzer detailed earlier on page 12. At the beginning of the feeding trial, 60 fish were randomly sampled, killed with

an overdose of anesthetic and pooled together. Six subsamples of a pooled sample were analyzed for initial carcass composition. At the end of the experiment, 15 fish from each replicate of dietary treatments were randomly collected, sacrificed with an overdose of the MS-222 and pooled separately. Three subsamples of the pooled samples were analyzed for final carcass composition.

Determination of RNA and DNA

RNA and DNA were determined by the method of Schneider (1957) as detailed earlier on page 13.

Evaluation of growth parameters

Calculation of various growth parameters was made according to the standard definitions as described under the General Methodology section (pages 13-14).

Statistical analyses

Statistical analyses of growth data were done using procedures as detailed earlier (Page 14). Dietary tryptophan requirement of fingerling *C. catla* was estimated by fitting the growth data to exponential analysis at 95% of maximum and minimum responses (Rodehutscord et al. 1997). The exponential model is described as follows:

$$Y=a\ (1-exp^{-k(x-xc)})$$

However, the equation employed for feed conversion ratio is as follows:

$$y = a+b*exp^{-kx}$$

Where x = dietary concentration of the tryptophan under test (% dry diet), a = plateau value of the respective curve, k = parameter characterizing the steepness of the curve, xc = dietary concentration of the tryptophan under test at y = 0, and b = maximum response to supplemented tryptophan. All the statistical analyses were done using Origin (version 6.1; Origin Software, San Clemente, CA).

RESULTS

Growth performance

Significantly higher absolute weight gain (AWG, 7.31 g/fish), best feed conversion ratio (FCR, 1.31) and highest protein gain (PG, 1.24 g/fish) values were obtained in group receiving the diet containing 0.23% tryptophan (Table 3). The relationship between above parameters and dietary tryptophan concentrations were evaluated by means of an exponential analysis at 95% maximum and minimum responses of AWG (Fig. 1), PG (Fig. 1) and FCR (Fig. 2) yielding the tryptophan requirement between 0.21-0.25% dry diet. The equations employed to calculate the dietary tryptophan requirement for each response variable are depicted in respective figures. Fish promptly accepted all diets and performed well throughout the experimental period. Feed intakes were similar amongst dietary treatments ($P>0.05$; Table 3) and ranged from 9.44-9.81 g/fish.

Carcass composition, RNA/DNA ratio and % survival

Dietary tryptophan significantly influenced the carcass composition (Table 4). Carcass protein improved ($P<0.05$) with the increasing concentrations of tryptophan up to 0.23% dry diet (T4). Further increase in dietary tryptophan (T5-T6) did not improve carcass protein. Carcass fat increased linearly with the increasing concentrations of dietary tryptophan in all the treatments. Moisture content decreased as the dietary tryptophan concentrations increased from 0.10-0.34% (T1-T6). Ash content remained constant ($P>0.05$) throughout the dietary treatments. The relationship of carcass fat and moisture content to the dietary tryptophan levels are depicted by the linear equations which are as under:

$$Y = 1.462 + 10.987\ X \quad (R^2 = 0.981) \text{ (Carcass fat)}$$

$$Y = 79.43 - 10.567\ X \quad (R^2 = 0.977) \text{ (Moisture)}$$

The RNA/DNA ratio was also affected by the varying levels of dietary tryptophan (Table 4). The exponential analysis of RNA/DNA ratio to dietary tryptophan concentrations projected the 95% maximum response ($Y_{95\%max}$) of RNA/DNA ratio at

0.21% dry diet. The equation that explained the relationship of RNA/DNA ratio to the dietary tryptophan concentrations is depicted in Figure 3. Lowest survival (73%) was recorded in fish fed at 0.10% dietary tryptophan (T1). It was 82% in fish fed diet containing 0.14% tryptophan (T2). However, a 100% survival was noted at 0.19 (T3), 0.23 (T4), 0.28 (T5) and 0.34% tryptophan of dry diets (T6).

Somatic indices

Relationship between dietary tryptophan levels and somatic indices including HSI, VSI and CF of fingerling *C. catla* are represented in Table 4. Fish receiving lowest level of dietary tryptophan (T1) had highest HSI value. Viscerosomatic index was found to decrease with the increase of dietary tryptophan up to 0.23% (T4) and, thereafter (T5 and T6), remained almost constant. Condition factor was found to correlate positively up to 0.23% dietary tryptophan (T4) beyond which (T5-T6) no significant change was noted.

Effect of tryptophan on pathological condition

Fish fed diets T1, T2 and T3 showed caudal fin erosion (Table 4). At the end of the feeding trial, 17.3% of fish fed 0.10% tryptophan of the dry diet (T1) exhibited caudal fin erosion. However, percentage of fish suffering from caudal fin erosion decreased from 17.3 to 2.6% as the concentrations of tryptophan increased from 0.10 (T1) to 0.19% (T3). However, in fish fed diets containing higher levels of tryptophan (T4-T6), no caudal fin erosion was recorded. No other pathological signs were recorded among the treatments during the length of the feeding trial.

Effect of tryptophan on hematological indices

Fish fed diet containing varying levels of tryptophan exhibited significant changes in hematological indices (Table 5). Hemoglobin (g/dl), hematocrit (%) and RBCs (10^6/mm^3) improved significantly ($P<0.05$) with the increasing concentrations of dietary tryptophan up to 0.23% (T4) and, remained nearly constant ($P<0.05$) thereafter.

DISCUSSION

Tryptophan is an essential dietary amino acid for fish. It is one of the most limiting amino acids in most of the plant proteins such as corn gluten meal (Anderson and Lall 2005; Abidi and Khan 2010b). It determines the efficiency of protein utilization and ultimately fish growth, and also affects the carcass quality of the fish. Therefore, it is necessary to determine the tryptophan requirement to optimize the growth performance of fish. Tryptophan requirement of fish has been estimated by adopting different statistical models in various studies (Rodehutscord et al. 1997; Gaylord et al. 2005; Farhat and Khan 2012b; Ahmed 2012a). The choice of a statistical model is important in interpreting the nutritional requirement experiments. Generally, non linear models are regarded as most appropriate for evaluating results from dose-response experiments because the response to improved dietary concentrations of a limiting nutrient is not linear (Cowey 1992; Rodehutscord et al. 1997). The efficiency of supplemented individual amino acids was found to decrease with the increasing dietary concentration of amino acids, resulting in plateaus that was described by exponential functions (Rodehutscord et al. 1997) and hence, dietary tryptophan requirement of fingerling *C. catla* was determined by the exponential analysis of AWG, FCR, PG, and RNA/DNA ratio against the dietary tryptophan concentrations (Fig. 1, 2 and 3). The high R^2 values obtained in this mathematical model suggest that this is a good model for analyzing the dietary tryptophan requirement. The use of the maximum (100%) response as the threshold level for an exponential function is not adequate. In order to deal with this, the use of 95% maximum response is a practical set point for estimating requirement with non-linear functions (Rodehutscord et al. 1997, NRC 2011). Thus, AWG, FCR, PG and RNA/DNA ratio were calculated at 95% of maximum and minimum responses using the exponential equations and the dietary tryptophan requirement was found to range between 0.21-0.25% dry diet, equivalent to 0.64-0.76% of dietary protein. The above tryptophan requirement of fingerling *C. catla* is higher than the requirement reported Asian sea bass *Lates calcarifer*, 0.41% (Coloso et al. 2004), but lower than that reported for *L. rohita* 1.13% (Murthy and Varghese 1997c), mrigal, *C. mrigala* 0.95% (Ahmed and Khan 2005a), rohu, *L. rohita* 0.90-0.95% (Abidi and Khan 2010b) and nearly similar to the

requirement of common carp, *C. carpio* 0.80% (Nose 1979); for rohu, *L. rohita* 0.59% (Khan and Jafri 1993) and hybrid striped bass *Morone chrysops x M. saxatilis* 0.60-0.70% (Gaylord et al. 2005) of the dietary protein. The tryptophan requirement of different species varies between 0.50 and 1.25% of the dietary protein (NRC 2011). Dietary tryptophan requirement of fingerling *C. catla* (0.64-0.76% dietary protein) determined in this study is lower than the requirement (0.95% dietary protein) reported by Ravi and Devaraj (1991) for the fry stage of this fish. Lower tryptophan requirement obtained in this study compared to higher tryptophan requirement reported by Ravi and Devaraj (1991) may be due to the differences in fish size as larger fish may have lower requirement (Sugiura et al. 2000). The variation in the tryptophan requirement of *C. catla* reported by Ravi and Devaraj (1991) with that obtained in this study may be because of the use of different response criteria as Ravi and Devaraj (1991) determined the tryptophan requirement on the basis of weight gain only whereas in this study, in addition to weight gain, the tryptophan requirement was determined on the basis of sensitive parameters such as protein gain, feed conversion ratio, RNA/DNA ratio and carcass composition. Use of mathematical models such as exponential used in this study and broken-line used by Ravi and Devaraj (1991) may be attributed to the variation in the tryptophan requirement of *C. catla*. Moreover, tryptophan requirement may also vary due to dietary protein levels fixed in this study (33%) and that taken (40%) by Ravi and Devaraj (1991). Disparity in the tryptophan requirements of various fish species may be due to the use of different feed ingredients in the experimental diets, dietary energy level of the test diets, feeding regime used and the environmental conditions in the culture system (Coloso et al. 2004; Abidi and Khan 2010b; Farhat and Khan 2012b). In addition, reference protein, size and age of fish, genetic differences, rearing conditions, assimilation rate, form of the amino acids and the energy obtained from feedstuff may also influence amino acid requirements of fish (Forster and Ogata 1998; Simmons et al. 1999; De Silva et al. 2000; Mai et al. 2006a).

The coating of crystalline L-amino acids reduces the absorption rate of the amino acids (Cho et al. 1992) and leaching (Alam et al. 2004) and also optimizes their utilization efficiency for protein gain. Ravi and Devaraj (1991) have used crystalline

amino acids without coating. However, in this study, crystalline amino acids were first coated with some amount of carboxymethyl cellulose followed by casein and gelatin which promotes the retention time of the amino acids in the gut leading to more efficient utilization of the ingested amino acids. It has also been reported that casein-coated supplementary amino acids in fish diets improve the utilization efficiency of amino acids by alleviating the relative absorption rate of certain amino acids in the gut (Murai et al. 1981). Thus, coating of crystalline amino acids led to the estimation of optimized tryptophan requirement of catla in this study than that reported by Ravi and Devaraj (1991).

Most authors have reported different tendency of growth of fish fed diets with excess levels of tryptophan; either it remained constant (Fagbenro and Nwanna 1999; Gaylord et al. 2005) or decreases when the dietary tryptophan concentration is higher than requirement (Murthy and Varghese 1997c; Ahmed and Khan 2005a; Abidi and Khan 2010b). In this study, weight gain was found to improve with the increased inclusion of tryptophan in diet from 0.10-0.23% (T1-T4) and remained nearly the same ($P>0.05$) in fish fed diets containing higher amount of tryptophan (T5 and T6) than T4. Absence of growth reduction at higher levels of dietary tryptophan noted in this study indicates that higher concentrations of dietary tryptophan did not adversely affect growth. This pattern of growth is almost similar to that reported by the other workers (Coloso et al. 1992; 2004; Fagbenro and Nwanna 1999; Gaylord et al. 2005). However, growth depressing effect at higher levels of dietary tryptophan has also been reported (Khan and Jafri 1993; Murthy and Varghese 1997c; Ahmed and Khan 2005a; Abidi and Khan 2010b).

Hepatosomatic index provides an indication on status of energy reserve (Balawi et al. 2011) and used as an important parameter in several amino acid requirements studies (Coloso et al. 2004; Espe et al. 2008; Ahmed 2012a; Farhat and Khan 2013a,b). In this study, fish fed tryptophan deficient diet showed significantly higher values of HSI which may be because of conversion of unutilized amino acids to energetic compounds stored in liver (Ballantyne 2001). A similar pattern of HSI as evident in this study was also reported in Asian sea bass (Coloso et al. 2004) and stinging catfish (Ahmed 2012a).

Assessment of the physiological and health status of fish may also be carried out by examining RNA/DNA ratio. The RNA is the precursor of protein synthesis and thus involved directly in somatic growth (Mustafa 1977; 1979; 1983; Mustafa and Jafri 1977; Mustafa and Mittal 1982; Mustafa and Zofair 1985; Bulow 1987; Mustafa et al. 1991; Abidi and Khan 2009). Therefore, any change in the RNA concentration will affect the growth rate or the physiological status of the organism directly (Elser et al. 1996). In this study, the highest RNA concentration and RNA/DNA ratio in fish fed 0.25% dietary tryptophan (T4) indicates that the fish were able to synthesize more RNA than those fed diets containing lower (T1, T2, T3) and higher concentrations of tryptophan (T5, T6). A significant reduction in DNA concentration of fish muscles was also noted with the increase in dietary tryptophan up to 0.25% (T4). This reduction in DNA concentration up to the above level is associated with the dilution of DNA concentration due to increase in volume of the cells leading to reduction in number of cells per unit weight of the tissue. Since DNA carries the genetic material in each cell and is present in the nucleus in fixed quantities (Love 1980), it is considered as an index of cell numbers contributing to unit weight of tissue. In fish losing weight, the size of cells decreases and thus number of cells contributing to unit weight of tissue increases, enhancing the number of nuclei and contributing to increased DNA concentration. In a weight-gaining fish, on the other hand, the DNA concentration becomes diluted with larger volume of cells per unit weight leading to reduction in number of cells per unit weight of the tissue. A positive correlation between RNA/DNA ratio and dietary amino acid up to requirement level as evident in this study have also been observed in previous studies (Di et al. 2009; Zehra and Khan 2013a,b,c).

The analysis of blood parameters is a precious indicator in evaluating the conditions of aquatic animals in response to stress, different sorts of pollutants, nutrition, ecological and physiological changes (Bahmani et al. 2001). Hematological parameters are most sensitive to dietary manipulations and are hence considered important tools in assessing the health of the fish. In the present study, RBCs, hemoglobin (Hb) and hematocrit (Ht%) values were affected by increasing tryptophan concentrations and found to be significantly higher at 0.25% dietary tryptophan (T4) indicating that the

THES

inclusion of tryptophan at this level is adequate to optimize the above hematological indices. Erythrocytes' membranes have a hydrophobic lipid bilayer with a protein skeletal meshwork attached to its inner surface by binding to integral (transmembrane) proteins, so when dietary amino acid deficiency occur, erythropoiesis is affected (Harvey 1997). Hence, fish fed deficient levels of dietary tryptophan (T1, T2, T3) showed lesser number of RBCs than that of the groups fed required amount of dietary tryptophan (T4) in this study. Lower values of hemoglobin (Hb) and hematocrit (Ht%) in fish fed T1, T2, T3 diets probably resulted from amino acid deficiencies suffered by fish fed tryptophan-deficient diets. Similar effects of feeding sub-optimum levels of dietary tryptophan on hematological indices were also reported in stinging catfish (Ahmed 2012a; Farhat and Khan 2012b).

Nutritional pathologies such as caudal fin erosion, scoliosis, lordosis, eye cataract, renal calcinosis, and short gill opercula have been observed in trout fed tryptophan-deficient diets (Kloppel and Post 1975; Poston and Rumsey 1983; Walton et al. 1984). Morphological abnormalities such as scoliosis and lordosis due to deficiency of tryptophan were noted in sockeye salmon (Halver 1957; Halver and Shanks 1960) and rainbow trout (Shanks et al. 1962; Poston and Rumsey 1983). Kloppel and Post (1975) reported that tryptophan deficient rainbow trout suffered from scoliosis and histological examinations revealed calcium deposits in the kidney. No nutritional deficiency signs except poor growth and caudal fin erosion were observed in fish fed tryptophan deficient diets. Fin erosion is a common pathological condition in fishes. At the end of this feeding trial, fish fed diets containing 0.10 (T1), 0.14 (T2) and 0.19% tryptophan (T3) exhibited 17.3, 5.3 and 2.6% caudal fin erosion, respectively. Similar effects of feeding diets containing deficient levels of tryptophan were also reported in rainbow trout (Poston and Rumsey 1983) and stinging catfish (Farhat and Khan 2012b).

Based on the exponential analysis of AWG, FCR, PG and RNA/DNA ratio against varying levels of dietary tryptophan, the requirement was found to range between 0.21-0.25% of dry diet, corresponding to 0.64-0.76% of dietary protein. Data generated during the present study would be useful to formulate tryptophan-balanced feeds for mass culture of this fish.

SUMMARY

A 12-week feeding trial was conducted in eighteen 70 L indoor polyvinyl circular troughs provided with a water flow-through system (1-1.5 L/min) at 28±1°C to evaluate the dietary tryptophan requirement of fingerling *Catla catla* (3.45±0.24 cm; 0.60±0.13 g). Six casein-gelatin based amino acid test diets (33% crude protein; 16.72 kJ/g gross energy) containing graded levels of L-tryptophan (0.10, 0.14, 0.19, 0.23, 0.28, 0.34% dry diet) were fed to triplicate groups of fish near to satiation at 08:00, 12:30 and 17:30h. Absolute weight gain, feed conversion ratio, protein gain, RNA/DNA ratio, hepatosomatic index, viscerosomatic index, condition factor and hematological indices improved with the increasing levels of tryptophan from 0.10 to 0.23% of dry diet. Significantly higher carcass protein was obtained at 0.23% tryptophan of the dry diet. Exponential analysis of absolute weight gain, feed conversion ratio, protein gain and RNA/DNA ratio against dietary tryptophan levels at 95% maximum and minimum responses displayed the tryptophan requirement at 0.25, 0.23, 0.25 and 0.21% dry diet, respectively. Inclusion of dietary tryptophan in the range of 0.21-0.25% dry diet, equivalent to 0.64-0.76% dietary protein is recommended in formulating tryptophan-balanced feed for the culture of this fish species.

Table 1 Composition of the basal diet

Ingredients	% dry diet
Casein[a] (vitamin and fat-free)	12.5
Gelatin[b]	4.17
Dextrin	33.55
Amino acid mixture[c]	20.55
Corn oil	5
Cod liver oil	2
Mineral mix[d,f]	4
Vitamin mix[e,f]	3
α- Cellulose	5.23
Carboxymethyl cellulose	10
Total	100
Digestible energy[g] (kJ/g, dry diet)	13.61
Calculated gross energy (kJ/g, dry diet)	16.72

[a]Crude Protein (76%); [b]Crude Protein (96%); [c]Amino acid mixture (g/100 g) arginine 1.321, histidine 0.221, isoleucine 1.897, leucine 1.707, lysine 1.191, methionine 0.914, cystine 0.74, phenylalanine 1.38, tyrosine 0.844, tryptophan 0, threonine 0.915, valine 1.495. alanine 1.10, aspartic acid 0.119, proline 0.901, glycine 5.797; (Loba Chemie, India); [d]Mineral mixture (g/100 g) calcium biphosphate 13.57; calcium lactate 32.69; ferric citrate 02.97; magnesium sulphate 13.20; potassium phosphate (dibasic) 23.98; sodium biphosphate 08.72; sodium chloride 04.35; aluminium chloride. $6H_2O$ 0.0154; potassium iodide 0.015; cuprous chloride 0.010; manganous sulphate. H_2O 0.080; cobalt chloride. $6H_2O$ 0.100; zinc sulphate. $7H_2O$ 0.40; [e]Vitamin mixture (g/100 g dry diet) choline chloride 0.500; inositol 0.200; ascorbic acid 0.100; niacin 0.003; calcium pantothenate 0.05; riboflavin 0.02; menadione 0.004; pyridoxine hydrochloride 0.005; thiamin hydrochloride 0.005; folic acid 0.0015; biotin 0.0005; alpha-tocopherol 0.04; vitamin B_{12} 0.00001; 2.072 g α-cellulose; choline chloride 0.500; inositol 0.200; ascorbic acid 0.100; niacin 0.003; calcium pantothenate 0.05; riboflavin 0.02; menadione 0.004; pyridoxine hydrochloride 0.005; thiamin hydrochloride 0.005; folic acid 0.0015; biotin 0.0005; alpha-tocopherol 0.04; vitamin B_{12} 0.00001; Loba Chemie, India; [f]Halver (2002); [g]Digestible energy was calculated on the basis of physiological fuel values 18.83, 14.64 and 35.56 kJ/g for protein, carbohydrate and fat, respectively (Jauncey 1982).

Table 2 Analyzed amino acid composition of the basal diet and whole chicken egg protein for fingerling *C. catla*[a]

Amino acid	Amino acid composition of basal diet (% dry diet)	Amino acid composition of 33% whole chicken egg protein
EAAs		
Arginine	2.14±0.08	2.11±0.04
Histidine	0.70±0.07	0.69±0.03
Isoleucine	2.68±0.02	2.64±0.05
Leucine	3.10±0.05	3.04±0.07
Lysine	2.41±0.02	2.38±0.02
Methionine	1.35±0.03	1.36±0.01
Phenylalanine	2.12±0.04	2.08±0.02
Threonine	1.46±0.05	1.43±0.02
Tryptophan	0.10±0.02	0.50±0.01
Valine	2.42±0.03	2.41±0.03
NEAAs		
Cystine	0.81±0.04	0.79±0.01
Tyrosine	1.51±0.05	1.49±0.04
Alanine	1.91±0.04	1.90±0.03
Aspartic acid	1.17±0.02	1.14±0.02
Glycine	6.11±0.03	4.27±0.11
Proline	2.80±0.07	2.77±0.06

[a]Determined by Hitachi L-8800 Automatic Amino Acid Analyzer

Table 3 Growth performance of fingerling *C. catla* fed diets with varying levels of tryptophan[a,b]

	Analyzed dietary tryptophan levels (%)					
	0.10 (T1)	**0.14 (T2)**	**0.19 (T3)**	**0.23 (T4)**	**0.28 (T5)**	**0.34 (T6)**
Average initial weight (g)	0.59±0.04[a]	0.60±0.08[a]	0.60±0.06[a]	0.59±0.05[a]	0.60±0.09[a]	0.60±0.07[a]
Average final weight (g)	2.98±0.12[d]	5.18±0.13[c]	6.87±0.15[b]	7.90±0.17[a]	7.74±0.11[a]	7.49±0.14[a]
Absolute weight gain (g/fish)	2.39±0.05[d]	4.58±0.04[c]	6.27±0.09[b]	7.31±0.06[a]	7.14±0.07[a]	6.89±0.03[ab]
Feed fed (g/fish)	9.44±0.12[a]	9.81±0.14[a]	9.79±0.16[a]	9.54±0.18[a]	9.64±0.14[a]	9.44±0.12[a]
Feed conversion ratio	3.95±0.09[a]	2.14±0.04[b]	1.56±0.07[c]	1.31±0.06[d]	1.35±0.04[d]	1.37±0.07[d]
Protein gain (g/fish)	0.29±0.01[d]	0.64±0.03[c]	1.04±0.02[b]	1.24±0.06[a]	1.18±0.04[a]	1.13±0.02[ab]

[a]Mean values of 3 replicates ± SEM. [b]Mean values sharing the same superscript are not significantly different (P>0.05)

Table 4 Carcass composition (wet basis), RNA/DNA ratio and somatic indices of fingerling *C. catla* fed diets with varying levels of tryptophan [a,b]

	Analyzed dietary tryptophan levels (%)						
	initial	0.10 (T1)	0.14 (T2)	0.19 (T3)	0.23 (T4)	0.28 (T5)	0.34 (T6)
Moisture (%)	78.27±0.28	78.41±0.63^{a}	77.98±0.65^{b}	77.52±0.67^{b}	76.85±0.61^{c}	76.29±0.68^{c}	76.02±0.66^{c}
Protein (%)	11.83±0.06	12.21±0.05^{c}	13.79±0.07^{b}	16.13±0.05^{a}	16.58±0.06^{a}	16.21±0.09^{a}	16.02±0.08^{a}
Fat (%)	3.27±0.11	2.71±0.02^{f}	2.98±0.06^{e}	3.39±0.08^{d}	3.84±0.04^{c}	4.69±0.05^{b}	5.23±0.03^{a}
Ash (%)	2.15±0.08	2.61±0.03^{a}	2.54±0.02^{a}	2.49±0.04^{ab}	2.63±0.03^{bc}	2.54±0.02^{c}	2.61±0.02^{d}
RNA (µg/100 mg dry weight basis)	682±3.74	678±4.15^{d}	748±4.62^{c}	1099±4.18^{b}	1126±4.91^{a}	1121±3.94^{a}	1116±4.15^{a}
DNA (µg/100 mg dry weight basis)	337±3.58	346±3.62^{a}	284±2.81^{b}	252±2.68^{c}	236±2.12^{d}	238±2.51^{d}	239±2.38^{d}
RNA/DNA ratio	2.02±0.04	1.96±0.02^{d}	3.65±0.07^{c}	4.36±0.05^{b}	4.78±0.06^{a}	4.71±0.06^{a}	4.66±0.02^{a}
Caudal fin erosion (%)	-	17.33±1.33^{a}	5.33±1.31^{b}	2.67±1.31^{c}	0	0	0
Viscerosomatic index (%)	6.48±0.02	6.48±0.02^{a}	5.92±0.05^{b}	4.63±0.08^{c}	4.41±0.09^{d}	4.48±0.06^{d}	4.51±0.05^{d}
Hepatosomatic index (%)	0.91±0.03	0.97±0.03^{a}	0.84±0.02^{b}	0.81±0.02^{b}	0.77±0.04^{b}	0.78±0.03^{b}	0.79±0.02^{b}
Condition factor	1.21±0.02	1.13±0.02^{d}	1.36±0.05^{c}	1.47±0.06^{b}	1.68±0.03^{a}	1.65±0.02^{a}	1.59±0.08^{a}
Survival (%)	-	73±0.68^{c}	82±0.75^{b}	100^{a}	100^{a}	100^{a}	100^{a}

[a]Mean values of 3 replicates ± SEM. [b]Mean values sharing the same superscript are not significantly different (P>0.05)

Table 5 Hematological parameters of fingerling *C. catla* fed diets with varying levels of tryptophan[a,b]

	Analyzed dietary tryptophan levels (%)					
	0.10 (T1)	0.14 (T2)	0.19 (T3)	0.23 (T4)	0.28 (T5)	0.34 (T6)
Hemoglobin (g/dl)	4.68±0.06[d]	6.25±0.05[c]	8.94±0.11[b]	10.83±0.09[a]	10.76±0.08[a]	10.69±0.09[a]
Hematocrit (%)	14.26±0.79[d]	19.48±0.84[c]	28.15±0.92[b]	30.84±0.71[a]	30.74±0.87[a]	30.21±0.81[a]
Red blood corpuscles (10^6/mm^3)	1.87±0.02[d]	2.34±0.03[c]	2.56±0.07[b]	2.89±0.04[a]	2.86±0.08[a]	2.82±0.05[a]

[a]Mean value of 3 replicates±SEM. [b]Mean values with the same superscripts in a row are insignificantly different (P>0.05)

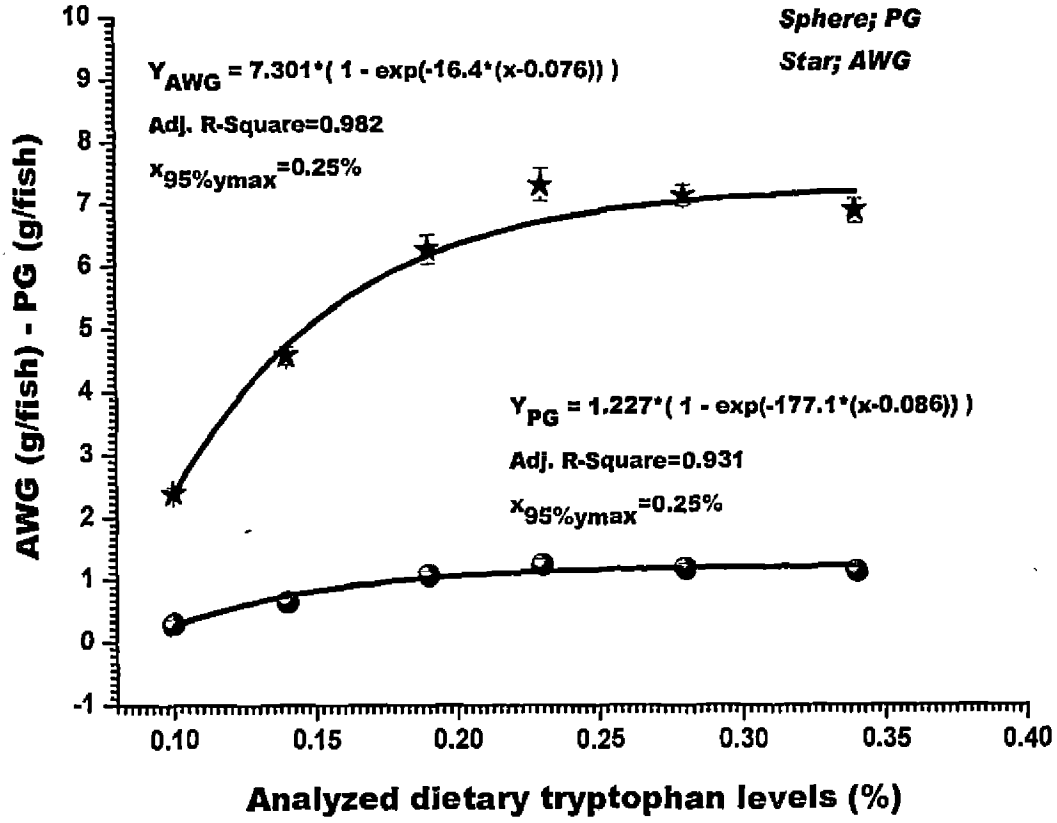

Fig. 1 Exponential relationship of dietary tryptophan to absolute weight gain and protein gain

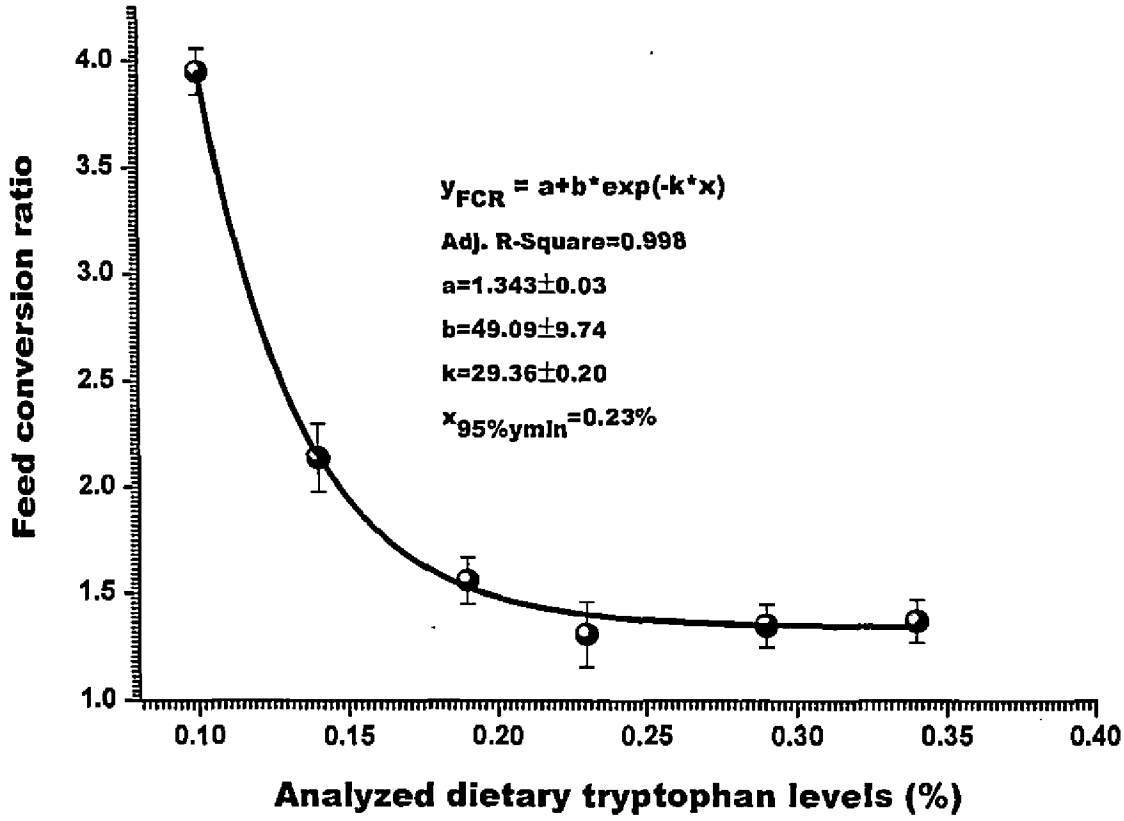

Fig. 2 Exponential relationship of dietary tryptophan to feed conversion ratio

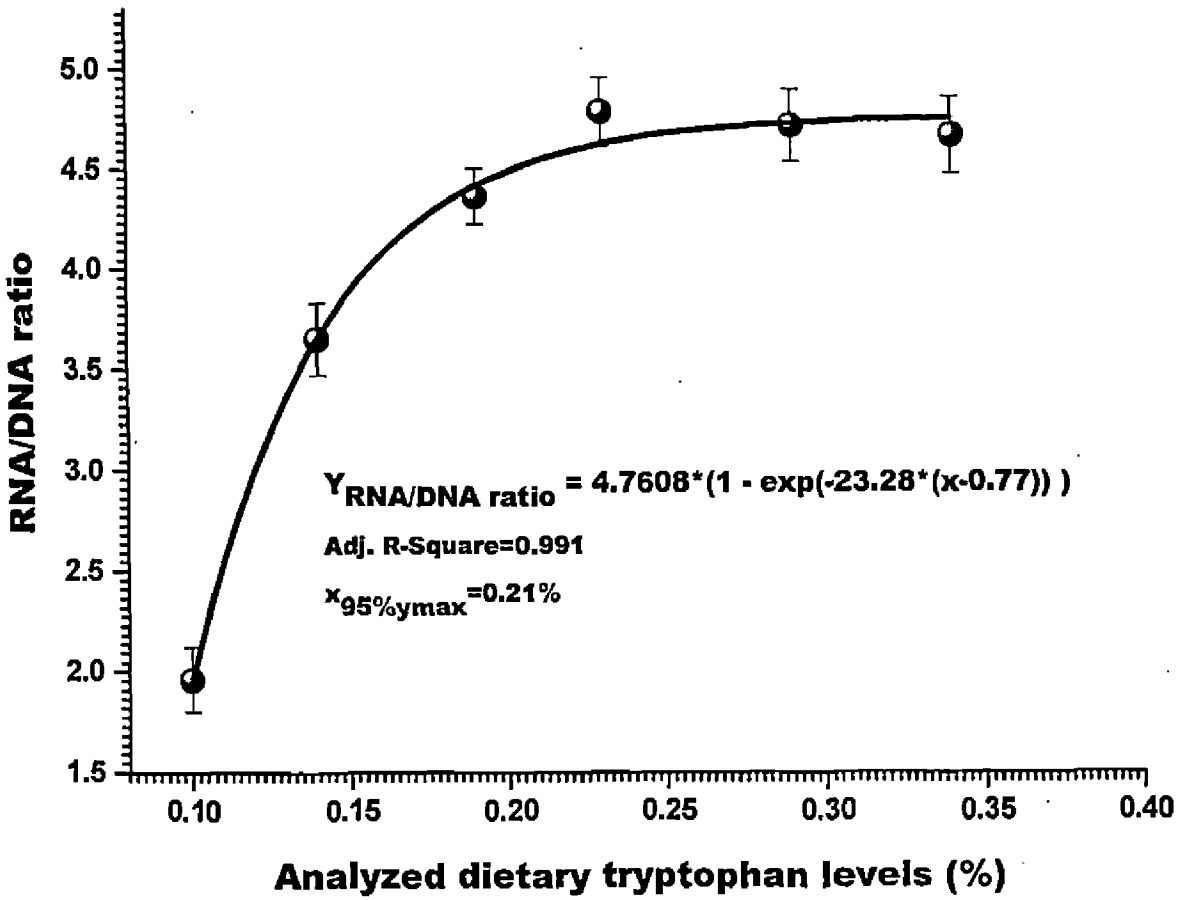

Fig. 3 Exponential relationship of dietary tryptophan to RNA/DNA ratio

CHAPTER 8

CHAPTER 8

DIETARY HISTIDINE REQUIREMENT OF FINGERLING *CATLA CATLA* (HAMILTON) BASED ON GROWTH, PROTEIN GAIN, HISTIDINE GAIN, RNA/DNA RATIO, HEMATOLOGICAL INDICES AND CARCASS COMPOSITION

INTRODUCTION

Indian major carps are the mainstay of Indian aquaculture requiring availability of high quality economical feeds for their culture. Among these carps, *Catla catla* is a highly esteemed carp and contributes to the bulk of freshwater fish catch. The fast growth together with its high nutritive value makes it an important food fish (Khan and Abidi 2008). For the future development of aquaculture of this species, it is of essence to develop quality feeds with all the essential nutrients. One such nutrient is histidine, an indispensable amino acid which plays a very important role in maintaining the osmoregulation process in fishes and also a role related to energy production. It is used in metabolic pathways during certain emergencies or harsh conditions (Abe and Ohmama 1987). It has vital catalytic roles in proteins, is a precursor for other bioactive chemicals and is an effector molecule in its own right. Many of these actions are mediated by the imidazole functional group (Schneider 1978). Because histidine and its related imidazole derivatives confer desirable taste and texture, dietary supplementation of histidine can improve sensory attributes of aquaculture sea foods (Ogata 2002). Moreover, dietary supplementation of histidine gives a higher quality fillet by enhancing intramuscular histidine concentration and pH which reduces muscle gapping in fish post-mortem (Forde-Skjaervik et al. 2006; Li et al. 2009). Due to various roles of histidine, inclusion of an optimal amount of dietary histidine is a prerequisite to the formulation of nutritionally-balanced feed for the successful aquaculture.

Several studies have been conducted on dietary histidine requirements of fishes such as common carp, *Cyprinus carpio* (Nose 1979), rainbow trout, *Oncorhynchus mykiss* (Ogino 1980), Nile tilapia, *Oreochromis niloticus* (Santiago and Lovell 1988),

rohu, *Labeo rohita* (Murthy and Varghese 1995; Abidi and Khan 2004b), European sea bass, *Dicentrarchus labrax*, gilthead seabream, *Sparus aurata* and turbot, *Scophthalmus maximus* (Kaushik 1998), mrigal, *Cirrhinus mrigala* (Ahmed and Khan 2005b), African catfish, *Clarias gariepinus* (Khan and Abidi 2009), Jian carp, *C. carpio* (Zhao et al. 2012c) and stinging catfish, *Heteropneustes fossilis* (Khan and Abidi 2012; Farhat and Khan 2013b). Although data on histidine requirement of fry size of *C. catla* exists (Ravi and Devaraj 1991), no information on histidine requirement of fingerling *C. catla* is available. Hence, this study was undertaken to optimize the histidine requirement of fingerling *C. catla* based on growth, protein gain, histidine gain, RNA/DNA ratio and carcass composition. Since RNA/DNA ratio is a sensitive tool in assessing the growth of the fish, it is also used to estimate the histidine requirement of the fingerling stage of this fish.

The study of the hematological characteristics of cultured fish species is an important tool in assessing the health status (Buentello et al. 2007; Khan and Abidi 2012; Farhat and Khan 2013b). The differences in blood cell formation and function are the indicators of dietary manipulations (Klinger et al. 1996). Histidine constitutes 10% amino acid composition of hemoglobin (Tristram and Smith 1963). Since hematological parameters were found to be sensitive to dietary manipulations (Mohammed and Sambo 2007), the changes in these parameters including hemoglobin, RBCs and hematocrit were also utilized to estimate the histidine requirement of this fish.

MATERIALS AND METHODS

Experimental diets

Six isonitrogenous (33% crude protein) and isocaloric (16.72 kJ/g gross energy) amino acid test diets using casein (fat-free), gelatin and crystalline L-amino acids with graded levels of histidine (0.25, 0.40, 0.55, 0.70, 0.85 and 1.00% dry diet) were formulated. The diets were marked as H1, H2, H3, H4, H5 and H6. Analyzed amino acid composition of experimental diets reflected the L-histidine content to be 0.25, 0.39, 0.53, 0.67, 0.83 and 0.96% of the dry diet. The levels of histidine in the amino acid test diets were fixed on

the basis of information available on other carps (NRC 2011). Crystalline L-amino acids excluding the test amino acid histidine were used to simulate the amino acid profile of the experimental diets to that of 33% whole chicken egg protein. The composition of basal diet is presented in Table 1. The basal diet used to quantify the histidine requirement of fingerling *C. catla* contained 0.25% dietary histidine from casein and gelatin. The amount of histidine was increased at the expense of glycine, on protein to protein basis to attain the intended concentrations of dietary histidine. The analyzed amino acid composition (% dry diet) of the basal diet is presented in Table 2. Method of preparation of experimental diets has been discussed under the General Methodology section (pages 9-10).

Experimental design and feeding trial

Source of the fish, their acclimation and details of the general experimental design has already been discussed under the General Methodology section (page 8).

Fingerling *C. catla* (3.65±0.15 cm; 0.65±0.36 g) were taken from the above acclimated fish lot and stocked in 70 L circular polyvinyl troughs (water volume 55 L) fitted with a continuous water flow-through (1-1.5 L/min) system in triplicate groups at the rate of 25 fish per trough for each dietary treatment. Fish were fed test diets in the form of dry crumbles (500 µm) to apparent satiation thrice a day at 08:00, 12:30 and 17:30h. Initial and weekly weights were recorded on a top-loading balance (Precisa 120A; 0.1 mg sensitivity, Oerlikon AG, Zurich, Switzerland) after anaesthetizing the fish with tricaine methane sulfonate (MS-222; 100 µg/ml). Fish were deprived of feed on the day they were weighed. The feeding trial lasted for 12 weeks. Faecal matter was siphoned before every feeding. Water quality indices were monitored daily during the feeding trial and were recorded following standard methods (APHA 1992). The range of water temperature, dissolved oxygen, free carbon dioxide, pH, total ammonia nitrogen, nitrites and total alkalinity based on daily measurements, was 27.9-28.3°C, 6.5-7.4 mg/L, 5.3-9.1 mg/L, 7.2-7.4, 0.29-0.35 mg/L, 0.03-0.09 mg/L and 68.8-81.3 mg/L, respectively.

Chemical analyses

Proximate composition of casein, gelatin, experimental diets, and initial and final carcass

was estimated using standard methods (pages 10-11). Gross energy content was determined on a Gallenkamp Ballistic Bomb Calorimeter as per the method described on page 12. Amino acid analysis of casein, gelatin, experimental diets, initial and final fish carcass was done using an automatic amino acid analyzer as detailed earlier (Page 12). At the beginning of the feeding trial, 60 fish were randomly sampled, killed and pooled together. Six subsamples of a pooled sample were analyzed for initial carcass composition. At the end of the experiment, 15 fish from each replicate of dietary treatments were randomly collected, sacrificed with an overdose of the MS-222 and pooled separately. Three subsamples of the pooled samples were analyzed for final carcass composition.

Hematological analyses

At the termination of the feeding trial, blood samples were collected in heparinized vials through cardiac puncture of the fish. The blood of five fish from each replicate of the treatment group was pooled to obtain enough samples for hematological analysis. Analysis of red blood cell counts, hemoglobin and hematocrit value were done as per the method detailed on page 88.

Lens quality analysis

At the end of the experiment, five fish from each replicate of the treatment were sampled, decapitated and the lens was removed aseptically from the left eye. The lenticular attachments were carefully removed and the lenses were mounted on a glass slide and observed carefully for cataract with the help of head-mounted binocular magnifier with in-built light source.

Determination of RNA and DNA

At the end of the feeding trial, white muscle was removed from those fish which were decapitated for the analysis of lens quality. Three subsamples of the muscle samples for each replicate of the treatment group were taken for the determination of RNA and DNA. RNA and DNA were determined by the method of Schneider (1957) as detailed earlier on

page 13.

Evaluation of growth parameters

Calculation of various growth parameters was made according to the standard definitions as described under the General Methodology section (pages 13-14).

Statistical analyses

Statistical analyses of growth data were done using procedures as detailed on page 14.

RESULTS

Absolute weight gain (AWG, g/fish), feed conversion ratio (FCR), protein gain (PG, g/fish), histidine gain (HG, mg/fish) and RNA/DNA ratio responded quadratically and improved significantly ($P<0.05$) with the increasing concentrations of dietary histidine up to 0.67% dry diet (Table 3). Fish fed at higher levels of dietary histidine (H5-H6) did not show significant changes ($P<0.05$) in above parameters. No significant ($P>0.05$) change in feed intake were observed in fish fed varying levels of dietary histidine (Table 3).

Quadratic regression analysis of AWG (Fig. 1), FCR, PG (Fig. 2), HG (Fig. 3) and RNA/DNA ratio against dietary concentrations of histidine reflected the histidine requirement between 0.63-0.73% dry diet, equivalent to 2.0-2.2% of the dietary protein. The equations employed to establish the quadratic relationship of each variable is as under:

$$Y_{AWG} = 2.825+29.1674X-18.506\ X^2\ (R^2=0.997),\ X_{95\%Ymax} =0.63\%$$

$$Y_{FCR} =5.8038-12.330X+8.128\ X^2\ (R^2=0.964),\ X_{95\%Ymax} =0.67\%$$

$$Y_{PG}=-0.6675+5.2676X-3.228\ X^2\ (R^2=0.998),\ X_{95\%Ymax} =0.66\%$$

$$Y_{HG} =-\ 24.977+185.775X-115.815X^2\ (R^2=0.995),\ X_{95\%Ymax} =0.66\%$$

$$Y_{RNA/DNA\ ratio} =-2.11551+16.155X-9.339\ X^2\ (R^2=\ 0.979),\ X_{95\%Ymax} =0.70\%$$

Table 4 reveals the effects of varying levels of dietary histidine on carcass composition. Carcass protein was found to improve with the increase of dietary histidine up to 0.67% (H4) beyond which a plateau was recorded. Carcass fat showed a positive correlation with the increase of dietary histidine. Moisture content was found to decrease with the increase of dietary histidine up to 0.67% (H4) and, thereafter, remained constant. No significant change in ash content were recorded in fish fed all the diets. HSI and VSI were negatively correlated with dietary histidine concentrations up to 0.67% (H4) beyond which no significant change in HSI and VSI was recorded. However, condition factor showed a positive correlation up to the above level of dietary histidine.

The hematological characteristics of fingerling *C. catla* fed diets containing graded levels of histidine are shown in Table 5. The hemoglobin concentration (4.06-9.61 g/dl), hematocrit (12.24-30.12%) and red blood cell counts (1.99-2.89 x 10^6/mm^3) significantly ($P<0.05$) improved with increasing dietary histidine from 0.25 (H1) to 0.67% (H4). No significant changes ($P>0.05$) in hematological parameters were noted at higher levels of dietary histidine (H5 and H6). The relationship between hematological parameters and dietary concentrations of histidine is described by the following quadratic equations;

$$Y_{Hb}=-2.5114+29.547X-18.1118\ X^2\ (R^2=0.978),\ X_{95\%Ymax}=0.66\%$$

$$Y_{Ht}=-7.187+87.167X-51.392\ X^2\ (R^2=0.971),\ X_{95\%Ymax}=0.68\%$$

$$Y_{RBCS}=1.009+4.442X-2.603\ X^2\ (R^2=0.985),\ X_{95\%Ymax}=0.63\%$$

The quadratic fitting of Hb (Fig. 4), Ht and RBCs against dietary histidine concentrations yielded the histidine requirement at 0.66, 0.68 and 0.63% dry diet, respectively.

No mortality was recorded in all the treatment groups. Visual inspection of lenses did not show evidence of cataract formation in this study. Lenses were clear and homogenous throughout the treatment groups.

DISCUSSION

The quadratic regression analysis of growth, feed conversion, protein gain, histidine gain, RNA/DNA ratio, hemoglobin, hematocrit and RBCs against dietary histidine exhibited the requirement in the range of 0.63-0.70% dry diet, equivalent to 1.91-2.12% of the dietary protein. The above histidine requirement worked out in this study is higher than the requirements reported for other fish species including coho salmon, *O. kisutch* 1.8% (Klein and Halver 1970); Japanese flounder, *Paralichthys olivaceus* 1.3% (Forster and Ogata 1998); red sea bream, *Chrysophrys major* 1.4% (Forster and Ogata 1998); turbot, *S. maxima* 1.5% (Kaushik 1998); rainbow trout, *O. mykiss* 1.6% (Ogino 1980); Nile tilapia, *O. niloticus* 1.7% (Santiago and Lovell 1988); gilthead seabream, *S. aurata* 1.7% (Kaushik 1998); European sea bass, *D. labrax* 1.6% (Kaushik 1998); African catfish, *C. gariepinus* 1.0-1.05% (Khan and Abidi 2009); but lower than that of Jian carp, *C. carpio* 2.4% (Zhao et al. 2012c) and comparable to the requirement of common carp, *C. carpio* 2.1% (Nose 1979); rohu, *L. rohita* 2.1% (Abidi and Khan 2004b) and mrigal, *C. mrigala* 2.1% (Ahmed and Khan 2005b) of dietary protein. The histidine requirement varies from 1.0 to 3.5% of dietary protein among species and within the species (NRC 2011). The variations in requirements might be related to the differences in age, feed allowance, experimental set up like water temperature, flow rate, stock density, protein source, digestibility, energy content of the diet, environmental and culture conditions (Tacon and Cowey 1985; Chiu et al. 1988; Akiyama et al. 1997; De Silva et al. 2000). The dietary histidine requirement of fingerling *C. catla* (1.91-2.12% of dietary protein) is lower than the requirement reported by Ravi and Devaraj (1991) for fry stage of *C. catla* (2.45% of dietary protein). The differences in histidine requirement worked out in this study on fingerling than those reported by Ravi and Devaraj (1991) on fry may probably be due to the variation in size of fish as smaller fish often have higher nutrient requirements than bigger ones due to its faster growth rates and utilizing amino acids as important source of energy in early stages (Ronnestad et al. 1999). Dietary protein content might also lead to differences in the amino acids requirements of fish (Coloso et al. 2004). Variation may also be attributed to different statistical or mathematical methods employed for the nutrient requirement estimates (Shearer 2000). Baker (1984) reported that the feeding

regime is one of the important factors bringing variations into the study. Moreover, reported differences in histidine requirement of catla in both the studies may be attributed to variation in response criteria. Histidine requirement reported by Ravi and Devaraj (1991) is based on weight gain only whereas in this study, in addition to weight gain, the histidine requirement is also based on the sensitive parameters such as protein gain, histidine gain, RNA/DNA ratio, carcass composition and hematological parameters. In addition to these, use of crystalline amino acid mixture as the only protein source in the study of Ravi and Devaraj (1991) might have also contributed to higher estimate of histidine requirement of catla than that of this study.

In the present study, a positive correlation of growth to the increasing concentrations of dietary histidine from 0.25 (H1) to 0.67% (H4) and stabilized growth performance at higher levels of histidine (H5-H6) is contrary to the reported growth-depressing effect of feeding higher amounts of histidine in common carp (Nose 1979), rohu (Murthy and Varghese 1995) and Jian carp (Zhao et al. 2012c). However, in the present study, insignificant fall in weight gain at higher levels of dietary histidine is similar to that reported on chum salmon (Akiyama et al. 1985).

A variety of methods have been used to evaluate the nutritional condition and growth of fish. Methods based on biochemical criteria have been considered more effective and sensitive because changes due to a feeding regime are first reflected at sub-cellular and cellular levels, and only thereafter in the whole organism (Robinson and Ware 1988). RNA and DNA contents of the fish have been used frequently as biochemical criteria (Mustafa 1977; 1979; 1983; Mustafa and Jafri 1977; Mustafa and Mittal 1982; Mustafa and Zofair 1985; Mustafa et al. 1991; Clemmesen et al. 1997; Cunha et al. 2003; Abidi and Khan 2009). RNA and RNA/DNA ratio is useful index of growth for both short-term (Bulow 1971) and long-term studies (Haines 1980; Bulow et al. 1981; Di et al. 2009; Zehra and Khan 2013a,b,c). Histidine is an abundant amino acid in plasma albumin of fish (Szebedinszky and Gilmour 2002). It participates in one-carbon unit metabolism, therefore, affecting RNA and protein synthesis (Li et al. 2009). In the present study, RNA/DNA ratio was found to improve with the increase of dietary histidine quadratically up to 0.70% indicating the best body protein synthesis at this level

of dietary histidine.

Significant improvement in carcass protein up to 0.67% dietary histidine (H4) in this study might be related to minimized amino acid catabolism. Higher carcass protein in fish fed H4 diet may be due to the availability of required amount of histidine in the diet. Zhao et al. (2012c) reported a marked decline in carcass protein content when fish were fed histidine-deficient diets or superfluous histidine diets, and suggested that the supplemental level of histidine should be optimized.

The condition factor quantifies fish condition and is an indicator of general fish health. A decline in the condition factor is usually interpreted as depletion of energy reserves such as stored liver glycogen or body fat (Adams et al. 1993). Somatic indices in fish have been reported to be influenced by dietary essential amino acids inclusion (Di et al. 2009; Zehra and Khan 2013a,b). In this study, HSI and VSI were found to decrease with the increasing concentrations of dietary histidine up to 0.67% (H4). However, condition factor improved with the increasing levels of dietary histidine up to the above level (H4) indicating the good health status of the fish receiving the diet H4. The higher values of HSI and VSI in fish fed diets containing lower levels of histidine may be due to more catabolism of amino acids, probably aiding energy deposition as fat in the liver and viscera.

Significance of hematological indices in relation to nutritional status of fish has been reported by several researchers (Buentello et al. 2007; Khan and Abidi 2012; Farhat and Khan 2013b). In this study, hematological indices such as hemoglobin, hematocrit and RBCs improved with the increase in dietary concentrations of histidine up to 0.67% (H4) indicating that dietary histidine seems to be adequate for the fish in maintaining growth and blood indices at this level of dietary histidine. Similar positive relationship between dietary histidine and hematological indices up to requirement level were also reported earlier in stinging catfish (Khan and Abidi 2012).

Histidine and histidine related compound show the property of anti-oxidation (Babizhayev et al. 2004), anti-inflammation (Wade and Tucker 1998), anti-glycation

(Hobart et al. 2004) and buffering capacity in the lens (Abe et al. 1985). Deficiency of these compounds causes the cataract (Bjerkas and Sveier 2004) which is a major problem for farmed fish (Bjerkas and Sveier 2004; Breck et al. 2005; Treasurer et al. 2007). Cataract due to deficiency of histidine has been reported by Breck (2004) and Breck et al. (2005) in Atlantic salmon. It causes a serious problem for fish farming industry with the potential economic loss and affected fish have reduced growth rates and increased susceptibility to secondary diseases compared to healthy fish (Menzies et al. 2002). Therefore, proper inclusion of dietary histidine is necessary. However, no cataract and any other pathological signs were observed in this study indicating that lower levels of dietary histidine were not effective in inducing cataract and pathological signs.

Based on regression analysis of AWG, PG, HG, RNA/DNA ratio, FCR, Hb, Ht and RBCs against dietary histidine concentrations, it is recommended that inclusion of dietary histidine in the range of 0.63-0.70% of the dry diet, equivalent to 1.91-2.12% of the dietary protein is essential in formulating histidine balanced commercial feeds for the culture of fingerling *C. catla*.

SUMMARY

To investigate the histidine requirement of fingerling *Catla catla* (3.65±0.15 cm; 0.65±0.36 g), six casein-gelatin based diets (33% CP; 13.54 kJ/g DE) containing graded levels of L-histidine (0.25, 0.39, 0.53, 0.67, 0.83, 0.96% analyzed histidine of the dry diet) were fed near to satiation thrice a day at 08:00, 12:30 and 17:30h for 12 weeks. Maximum absolute weight gain (AWG; 8.63 g/fish), protein gain (PG; 1.45 g/fish), histidine gain (HG, 48.19 mg/fish), RNA/DNA ratio (4.15), best feed conversion ratio (FCR; 1.31), highest hemoglobin (Hb, 9.61 g/dl), RBCs (2.84 x 10^6/mm^3) and hematocrit (Ht, 30.12%) were recorded in fish fed diet containing 0.67% histidine. However, quadratic regression analysis of AWG, PG, HG, RNA/DNA ratio, FCR, Hb, Ht and RBCs against dietary histidine at 95% maximum and minimum response reflected the histidine requirement at 0.63, 0.66, 0.66, 0.70, 0.67, 0.66, 0.68 and 0.63% dry diet, respectively. Carcass protein was found to improve significantly ($P<0.05$) from 13.36 to 16.42% with the increase of dietary histidine from 0.25 to 0.67%. Based on regression

analysis of AWG, PG, HG, RNA/DNA ratio, FCR, Hb, Ht and RBCs, it is recommended that the diet for fingerling catla should contain histidine in the range of 0.63-0.70% dry diet, equivalent to 1.91-2.12% of the dietary protein for optimum growth, feed utilization, blood profile and carcass composition.

Table 1 Composition of the basal diet

Ingredients	Basal diet (%)
Casein[a] (fat-free)	8.7
Gelatin[b]	2.9
Dextrin	33.38
Amino acid mixture[c]	25.32
Corn oil	5
Cod liver oil	2
Mineral mix[d,f]	4
Vitamin mix[e,f]	3
α- Cellulose	5.70
Carboxymethyl cellulose	10
Total	100
Analyzed crude protein	33.15
Digestible energy[g] (kJ/g, dry diet)	13.54
Calculated gross energy (kJ/g, dry diet)	16.72

[a]Crude Protein (76%); [b]Crude Protein (96%); [c]Amino acid mixture (%) arginine 1.561, histidine 0, isoleucine 1.849, leucine 2.129, lysine 1.552, methionine 1.051, cystine 0.755, phenylalanine 1.593, tyrosine 1.041, threonine 1.072, tryptophan 0.430, valine 1.774. alanine 1.343, aspartic acid 1.054, glutamic acid 0.223, proline 1.469, glycine 6.531; serine 0.135 (Loba Chemie, India); [d]Mineral mixture (g/100g of mineral mix) calcium biphosphate 13.57; calcium lactate 32.69; ferric citrate 02.97; magnesium sulphate 13.20; potassium phosphate (dibasic) 23.98; sodium biphosphate 08.72; sodium chloride 04.35; aluminium chloride. $6H_2O$ 0.0154; potassium iodide 0.015; cuprous chloride 0.010; manganous sulphate. H_2O 0.080; cobalt chloride. $6H_2O$ 0.100; zinc sulphate. $7H_2O$ 0.40. [e]Vitamin mixture (g/100 g dry diet) choline chloride 0.500; inositol 0.200; ascorbic acid 0.100; niacin 0.075; calcium pantothenate 0.05; riboflavin 0.02; menadione 0.004; pyridoxine hydrochloride 0.005; thiamin hydrochloride 0.005; folic acid 0.0015; biotin 0.0005; alpha-tocopherol 0.04; vitamin B_{12} 0.00001; 2 g α-cellulose; [f]Halver (2002); Loba Chemie, Mumbai, India; [g]Digestible energy was calculated on the basis of physiological fuel values 18.83, 14.64 and 35.56 kJ/g for protein, carbohydrate and fat, respectively (Jauncey 1982).

Table 2 Analyzed amino acid composition of the whole chicken egg protein and basal diet for fingerling *C. catla*[a,b]

Amino acid	Amino acid composition of 33% whole egg protein	Initial analyzed amino acid composition of basal diet (%)
EAAs		
Arginine	2.11±0.06	2.10±0.08
Histidine	0.69±0.04	0.25±0.07
Isoleucine	2.64±0.03	2.68±0.02
Leucine	3.04±0.08	3.06±0.05
Lysine	2.38±0.07	2.35±0.02
Methionine	1.36±0.03	1.38±0.03
Phenylalanine	2.08±0.09	2.06±0.04
Threonine	1.43±0.04	1.47±0.05
Tryptophan	0.50±0.02	0.51±0.02
Valine	2.41±0.03	2.42±0.03
NEAAs		
Cystine	0.79±0.02	0.82±0.04
Tyrosine	1.49±0.02	1.51±0.05
Alanine	1.90±0.01	1.89±0.04
Aspartic acid	1.14±0.02	1.16±0.02
Glycine	4.27±0.04	10.07±0.03
Proline	2.77±0.03	2.81±0.07

[a]Determined by Hitachi L-8800 Automatic Amino Acid Analyze, [b]Mean values of 5 replicates ± SEM.

Table 3 Growth performance of fingerling *C. catla* fed diets with varying levels of histidine[a,b]

	Analyzed dietary histidine levels (%)					
	0.25 (H1)	0.39 (H2)	0.53 (H3)	0.67 (H4)	0.83 (H5)	0.96 (H6)
Average initial weight (g)	0.64±0.02[a]	0.66±0.05[a]	0.65±0.02[a]	0.65±0.03[a]	0.64±0.07[a]	0.64±0.08[a]
Average final weight (g)	3.92±0.03[d]	6.52±0.06[c]	8.50±0.09[b]	9.29±0.09[a]	9.17±0.11[a]	9.12±0.09[a]
Absolute weight gain (g/fish)	3.28±0.02[d]	5.86±0.04[c]	7.85±0.08[b]	8.63±0.07[a]	8.53±0.02[a]	8.48±0.05[a]
Feed conversion ratio	3.37±0.03[a]	2.01±0.05[b]	1.49±0.02[c]	1.31±0.02[d]	1.31±0.04[d]	1.34±0.02[d]
Feed intake	11.05±0.08[a]	11.77±0.12[a]	11.69±0.07[a]	11.31±0.11[a]	11.17±0.13[a]	11.36±0.11[a]
Protein gain (g/fish)	0.45±0.01[d]	0.88±0.01[c]	1.23±0.03[b]	1.45±0.03[a]	1.43±0.04[a]	1.42±0.03[a]
Histidine gain (mg/fish)	14.45±0.26[d]	29.05±0.37[c]	43.01±0.42[b]	48.19±0.49[a]	47.64±0.51[a]	47.36±0.54[a]
RNA/DNA ratio	1.43±0.06[d]	2.69±0.01[c]	3.56±0.02[b]	4.85±0.02[a]	4.82±0.04[a]	4.74±0.03[a]

[a]Mean values of 3 replicates ± SEM. [b]Mean values sharing the same superscripts in the same row are insignificantly different ($P>0.05$).

Table 4 Carcass composition (%wet basis) and somatic indices of fingerling *C. catla* fed diets with varying levels of histidine[a,b]

	Analyzed dietary histidine levels (%)						
	Initial	**0.25 (H1)**	**0.39 (H2)**	**0.53 (H3)**	**0.67 (H4)**	**0.83 (H5)**	**0.96 (H6)**
Moisture%	79.25±1.4	78.16±1.38^{a}	77.25±1.51^{b}	76.32±1.42^{c}	74.13±1.13^{d}	75.11± 1.29^{d}	75.28± 1.24^{d}
Protein%	11.81±0.8	13.36±0.15^{d}	14.62±0.13^{c}	15.37±0.16^{b}	16.42±0.15^{a}	16.41±0.12^{a}	16.38±0.16^{a}
Fat%	4.61±0.05	3.43±0.08^{f}	3.98±0.06^{e}	4.47± 0.05^{d}	4.91±0.06^{c}	5.52± 0.07^{b}	5.96± 0.05^{a}
Ash%	2.75±0.04	2.68±0.02^{a}	2.65± 0.03^{a}	2.67±0.04^{a}	2.67±0.03^{a}	2.64± 0.02^{a}	2.68± 0.03^{a}
Viscerosomatic index (%)	5.16±0.11	6.72±0.13^{a}	6.14±0.12^{b}	5.62±0.15^{c}	5.11±0.08^{d}	5.13±0.09^{d}	5.17±0.07^{d}
Hepatosomatic index (%)	0.92±0.02	1.08±0.04^{a}	0.97±0.03^{b}	0.93±0.02^{c}	0.89±0.04^{cd}	0.91±0.03^{c}	0.92±0.05^{c}
Condition factor	1.16±0.02	1.09±0.02^{d}	1.21±0.04^{c}	1.42±0.02^{b}	1.58±0.03^{a}	1.55±0.02^{a}	1.53±0.02^{a}

[a]Mean value of 3 replicates ± SEM. [b]Mean values sharing the same superscripts in the same row are insignificantly different (P>0.05

Table 5 Hematological parameters of fingerling *C. catla* fed diets with varying levels of histidine[a,b]

	Analyzed dietary histidine levels (%)					
	0.25 (H1)	**0.39 (H2)**	**0.53 (H3)**	**0.67 (H4)**	**0.83 (H5)**	**0.96 (H6)**
Hemoglobin (g/dl)	4.06±0.01[d]	5.81±0.07[c]	7.96±0.08[b]	9.61±0.11[a]	9.45±0.09[a]	9.12±0.13[a]
Hematocrit (%)	12.24±0.26[d]	17.91±0.29[c]	23.26±0.32[b]	30.12±0.31[a]	29.81±0.35[a]	28.65±0.32[a]
Red blood corpuscles (10^6/mm^3)	1.99±0.01[d]	2.29±0.02[c]	2.61±0.05[b]	2.89±0.04[a]	2.88±0.04[a]	2.87±0.06[a]

[a]Mean value of 3 replicates±SEM. [b]Mean values with the same superscripts in a row are insignificantly different (P>0.05)

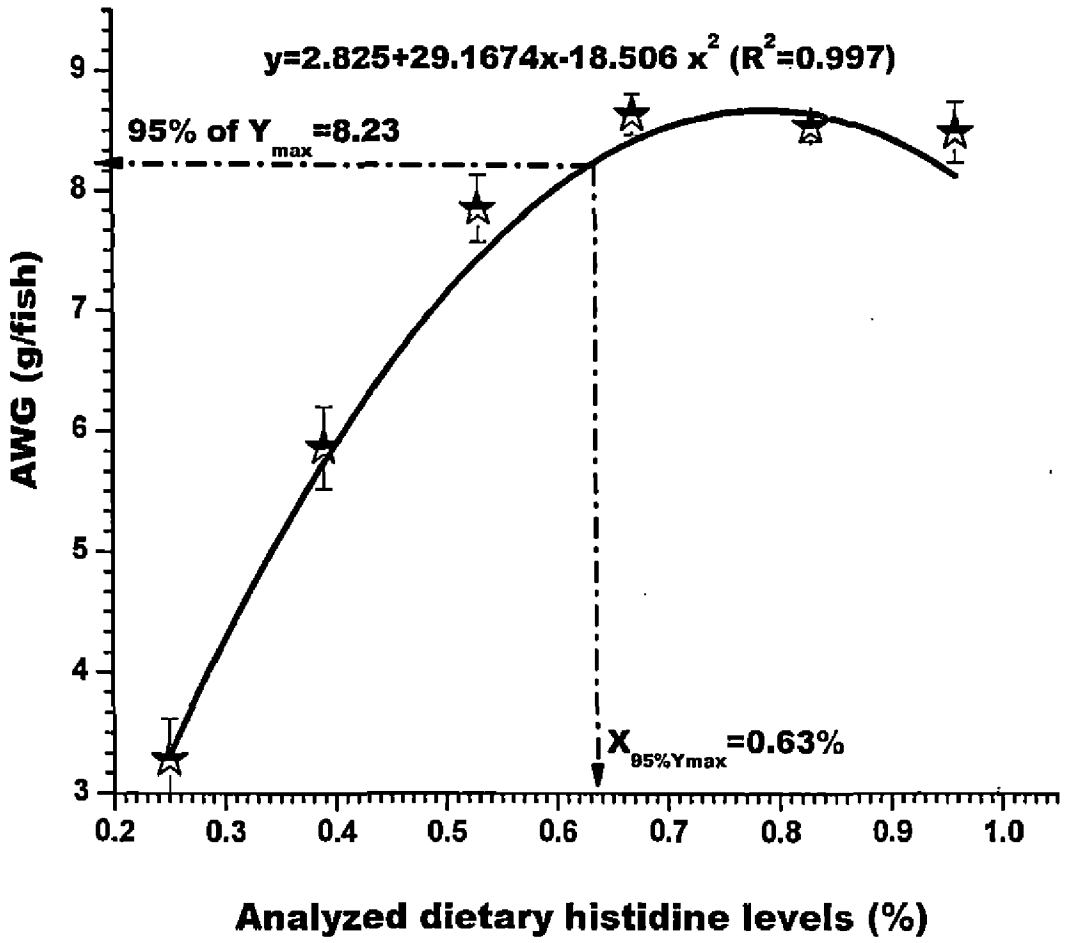

Fig. 1 Quadratic relationship of dietary histidine to absolute weight gain

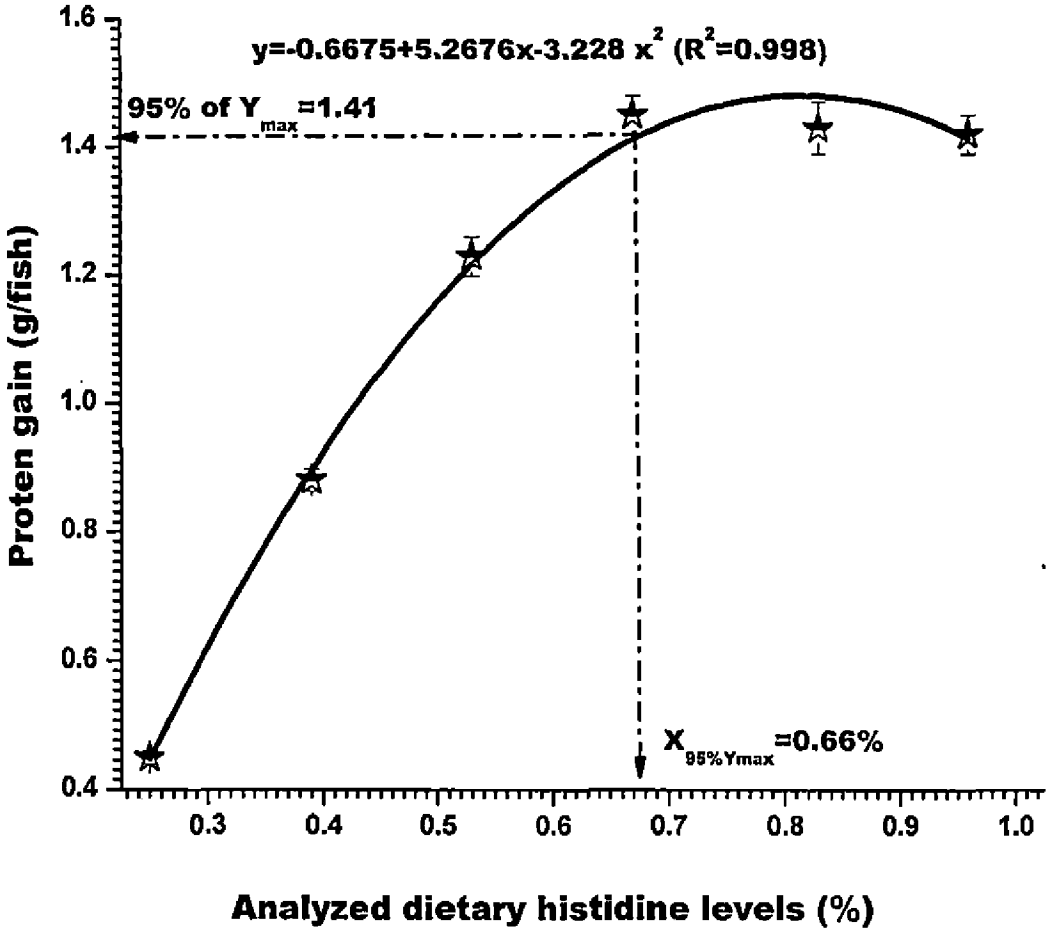

Fig. 2 Quadratic relationship of dietary histidine to protein gain

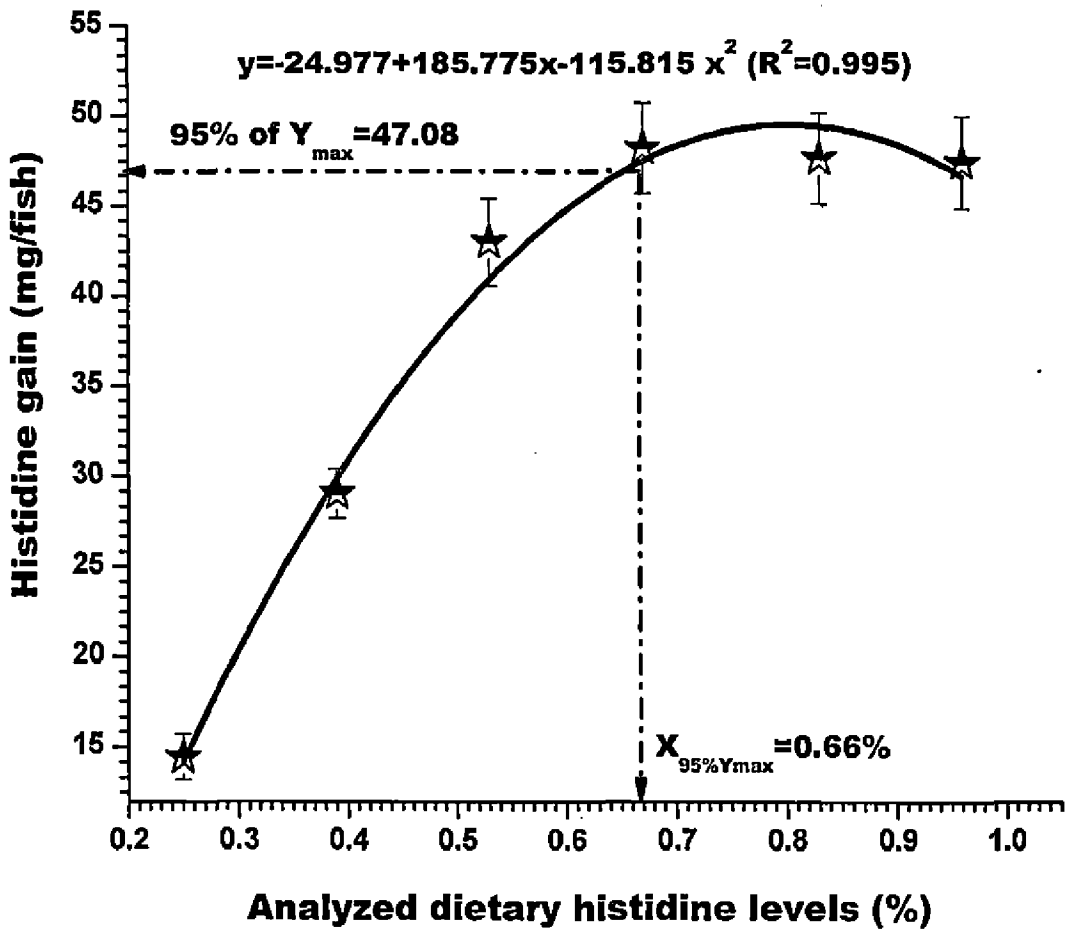

Fig. 3 Quadratic relationship of dietary histidine to histidine gain

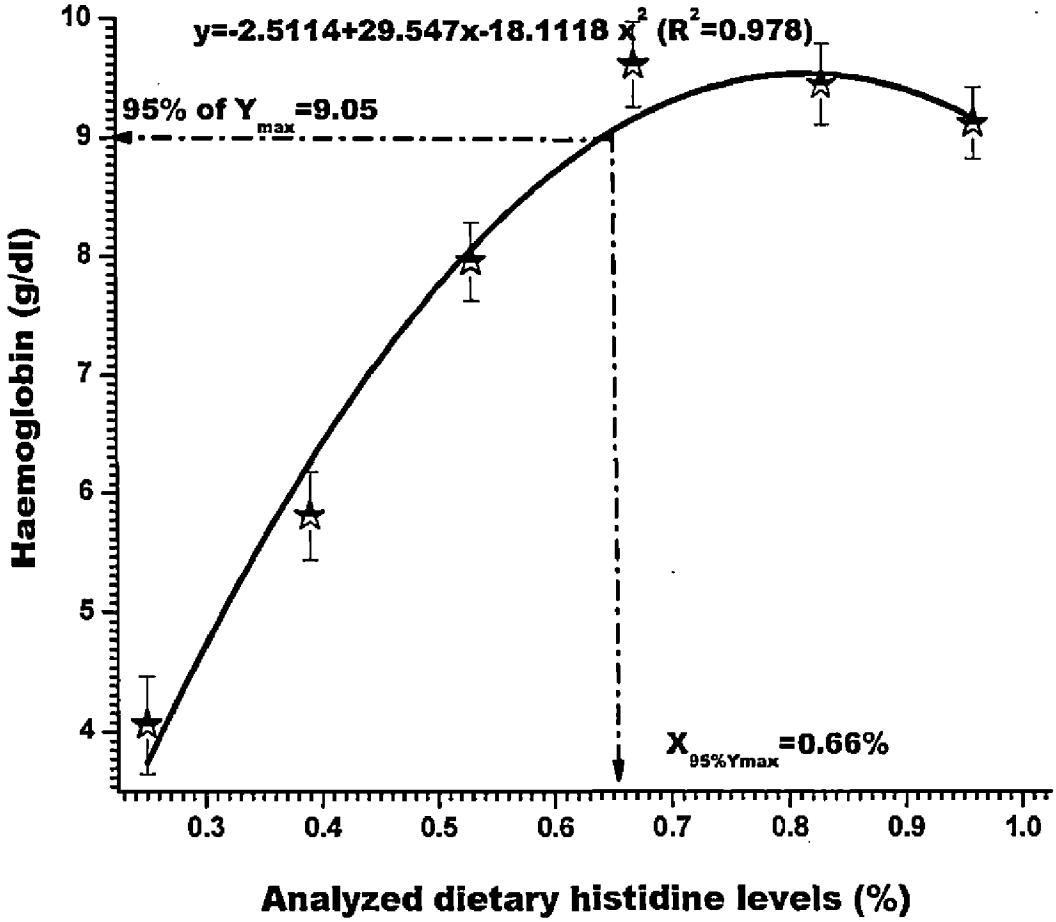

Fig. 4 Quadratic relationship of dietary histidine to hemoglobin content

CHAPTER 9

CHAPTER 9

TOTAL SULPHUR AMINO ACID REQUIREMENT AND MAXIMUM REPLACEMENT VALUE OF METHIONINE BY CYSTINE FOR FINGERLING *CATLA CATLA* (HAMILTON)

INTRODUCTION

Fish requires high quality nutritionally balanced feeds for growth and attainment of market size within the shortest possible time (Gabriel et al. 2007). Development and management of fish feed plays a vital role in aquaculture growth and expansion. It is a major factor that determines the profitability of aquaculture venture (Gabriel et al. 2007). According to Erondu et al. (2006), fish feed consist of 60% production cost and the protein component is to be the most expensive in terms of overall feed cost. Dietary protein is essential for the growth and development of fish because it provides essential amino acids. Fish generally have a higher protein requirement than land animals (Lovell 1989). The nutritive value of any protein depends primarily on its capacity to satisfy the needs of indispensable amino acids (Bhushan 1991). Amino acid represents the most important constituent of fish feeds (NRC 2011). They are not only building blocks for tissue proteins but are also potent antioxidants, regulators of hormone secretion and cell signalling molecules (Wang et al. 2007). The quantity and quality of dietary amino acid have a pronounced effect on growth rate, efficiency of feed conversion and carcass composition of fish (Jena et al. 1998). Therefore, it is crucial to find out the essential amino acid requirements so that the dietary needs for these could be satisfied.

Methionine is one of the most limiting amino acids in many fish diets especially those containing plant protein sources such as soybean meal, peanut meal, copra meal, leucaena leaf meal, or cassava leaf meal (Coloso et al. 1999). Supplementing soybean meal with commercially available methionine has been shown to improve growth response of many fish species (Viola et al. 1982; Murai et al. 1989; Cai and Burtle 1996). For the supplementation of methionine in fish feeds containing methionine-deficient plant protein sources, accurate information on methionine requirement of fish is essential.

Methionine is an indispensable amino acid which along with the dispensable amino acid cystine constitutes the total sulfur amino acids (NRC 2011). It is known as a precursor of choline and various other metabolic processes (Ruchimat al. 1997; Kasper et al. 2000). As cystine can only be synthesized from a methionine precursor, a portion of the methionine requirement can be spared by cystine in some fish species (Moon and Gatlin 1991; Kim et al. 1992; Griffin et al. 1994; Goff and Gatlin 2004). So, it is important to consider the dietary cystine concentration to quantify the total sulphur amino acid requirement of the cultured species for maximum growth and efficient feed utilization (Luo et al. 2005).

The essentiality of total sulphur amino acid (TSAA) to support maximum growth have been reported in several cultured fish species such as rohu *Labeo rohita* (Abidi and Khan 2011); common carp *Cyprinus carpio* (Xiao et al. 2011); mrigal *Cirrhinus mrigala*; rainbow trout *Oncorhynchus mykiss*; Japanese flounder *Paralichthys olivaceus*; red drum *Sciaenops ocellatus*; Arctic charr *Salvelinus alpinus*; Asian sea bass *Lates calcarifer*; channel catfish *Ictalurus punctatus*; European sea bass *Dicentrarchus labrax*; hybrid striped bass *Morone chrysops x M. saxatilis*; Mossambique tilapia *Oreochromis mossambicus*; cobia *Rachycentron canadum*; Atlantic Salmon *Salmo salar*; yellow perch *Perca flavescens*; yellow croaker *Pseudosciaena crocea* (NRC 2011); black sea bream *Sparus macrocephalus* (Zhou et al. 2011b) and stinging catfish *Heteropneustes fossilis* (Ahmed 2012b). Although data exists with regards to the dietary methionine requirements of fry stage of *Catla catla* (Ravi and Devaraj 1991), information on TSAA requirement and cystine replacement value for methionine of fingerlings *C. catla* is completely lacking. This study was, therefore, aimed to investigate the TSAA requirement and cystine replacement value for methionine for the fingerling stage of this fish species.

MATERIALS AND METHODS

Experimental diets

Two separate experiments were conducted to determine the total sulphur amino acid

requirement (experiment I) and the cystine replacement value for methionine (experiment II) in fingerling *C. catla*. For conducting experiment I, six isonitrogenous (33% crude protein) and isocaloric (16.72 kJ/g gross energy) amino acid test diets containing casein (vitamin and fat-free), gelatin and crystalline L-amino acids with graded levels of methionine (0.50, 0.75, 1.00, 1.25, 1.50 and 1.75% dry diet) were formulated (Table 1). Diets were designated as D1, D2, D3, D4, D5 and D6. The amount of methionine contributed by casein and gelatin in basal diet (D1) was 0.48 and 0.02%, respectively. To make the intended concentrations of dietary methionine in the amino acid test diets, the amount of methionine was increased at the expense of glycine on protein basis. Experimental diets contained 0.06% cystine contributed by casein which is almost negligible. The resulting TSAA levels in different experimental diets were 0.56, 0.81, 1.06, 1.31, 1.56 and 1.81% dry diet. Amino acid analysis of diets revealed the L-TSAA content to be 0.55, 0.79, 1.07, 1.30, 1.53 and 1.79% of the dry diet. The levels of TSAA in the amino acid test diets were fixed on the basis of information available on other carps (NRC 2011). The analyzed amino acid composition of the basal diet is presented in Table 2. In Experiment II, replacement value of cystine for methionine was determined by feeding six diets (R1, R2, R3, R4, R5 and R6) containing various ratios of L-methionine and L-cystine (80:20, 70:30, 60:40, 50:50, 40:60, 30:70) on equimolar sulphur basis (Table 3). The level of TSAA was fixed at 1.28% of the dry diet as per the requirement determined in experiment I. The amount of methionine and cystine contributed by the intact protein sources in the experimental diets was 0.34 and 0.04%, respectively. Crystalline methionine and cystine at 0.64 and 0.26% were added in experimental diet to provide the first replacement level (R1). The diets were made isonitrogenous by adjusting the level of glycine. The analyzed amino acid composition of the experimental diet (R1) used in experiment II is provided in Table 4. Crystalline L-amino acids excluding the test amino acids methionine and cystine were used to simulate the amino acid profile of the experimental diets to that of 33% whole chicken egg protein. Method of preparation of experimental diets has been discussed under the General Methodology section (pages 9-10).

Experimental design and feeding trials

Source of the fish, their acclimation and details of the general experimental design has already been discussed under the General Methodology section (page 8).

For conducting experiment I, fingerling *C. catla* (3.55±0.06 cm; 0.65±0.02 g) were taken from the above acclimated fish lot and stocked in triplicate groups in 70 L circular polyvinyl troughs (water volume 55 L) fitted with a continuous water flow-through (1-1.5 L/min) system at the rate of 25 fish per trough for each dietary treatment level. Fish were fed test diets in the form of dry crumbles (500 µm) to apparent satiation thrice a day at 08:00, 12:30 and 17:30h. Initial and weekly weights were recorded on a top-loading balance (Precisa 120A; 0.1 mg sensitivity, Oerlikon AG, Zurich, Switzerland) after anaesthetizing the fish with tricaine methane sulfonate (MS-222; 100 µg/ml). Fish were deprived of feed on the day they were weighed. The feeding trial lasted for 12 weeks. Faecal matter was siphoned before every feeding. For conducting experiment II, fingerling *C. catla* of almost similar size (3.65±0.08 cm; 0.67±0.04 g) were selected. Feeding trial was conducted following the same procedures as used in experiment I. Water quality indices were monitored during the feeding trial and were recorded following standard methods (APHA 1992). The range of water temperature, dissolved oxygen, free carbon dioxide, pH, total ammonia nitrogen, nitrites and total alkalinity based on daily measurements, was 25.3-28.5°C, 6.1-7.0 mg/L, 6.4-10.5 mg/L, 7.4-7.3, 0.28-0.32 mg/L, 0.05-0.08 mg/L and 61.5-76.9 mg/L, respectively.

Sample collection and chemical analyses

At the beginning of the feeding trial, 60 fish were randomly sampled, killed and pooled together. Six subsamples of a pooled sample were analyzed for initial carcass composition. At the end of the experiment, 15 fishes from each replicate of dietary treatments were randomly collected, sacrificed with an overdose of MS-222 and pooled separately. Three subsamples of the pooled samples were analyzed for final carcass composition. At the end of the 12-week feeding trial, five fish from each replicate of the treatment were anesthetized with MS-222 (Tricaine Methane Sulphonate; 100 µg/ml)

before taking the body measurements. Liver and viscera of each specimen was carefully removed and weights of fish, viscera and liver were used to calculate viscerosomatic index (VSI), hepatosomatic index (HSI) and condition factor (CF). Proximate composition of casein, gelatin, experimental diets, and initial and final carcass was estimated using standard methods (pages 10-11). Gross energy content was determined on a Gallenkamp Ballistic Bomb Calorimeter as per the method described on page 12. Amino acid analysis of casein, gelatin, experimental diets, initial and final fish carcass was done using an automatic amino acid analyzer as detailed earlier (page 12).

Evaluation of growth parameters

Calculation of various growth parameters was made according to the standard definitions as described under the General Methodology section (pages 13-14).

Statistical analyses

Statistical analyses of growth data were done using procedures as detailed earlier (page 14).

RESULTS

Experiment I: TSAA requirement

Table 5 summarizes the data relating to growth performance of fish fed diets containing varying levels of TSAA. The growth parameters including absolute weight gain (AWG,) protein efficiency ratio (PER), feed efficiency (FE), protein gain (PG) and total sulphur amino acid gain (TSAAG) were found to improve with the increasing concentrations of dietary TSAA up to 1.31% (D4) and then leveled off. The dietary TSAA requirement of fingerling *C. catla* was determined by calculating the quadratic equations at 95% maximum response ($Y_{95\%max}$). The quadratic regression analysis of AWG and PER yielded the TSAA requirement at 1.29, and 1.28% dry diet, corresponding to 3.91 and 3.88% dietary protein (Fig. 1). When PG and FE were plotted against dietary TSAA levels, $Y_{95\%max}$ was obtained at 1.29 and 1.30% dry diet, corresponding to 3.91 and 3.94%

dietary protein (Fig. 2). Quadratic regression analysis of the TSAAG at 95% maximum response projected that this response variable was best attained at 1.27% TSAA of the dry diet, corresponding to 3.85% dietary protein (Fig. 3). No significant differences existed between feed intake and dietary TSAA concentrations (Table 5). Fish promptly accepted the experimental diets from the beginning and maintained normal behaviour throughout the experimental period. The quadratic equations for above parameters employed to calculate the TSAA requirements are given in their respective figures.

Carcass composition of fingerling *C. catla* was influenced by varying concentrations of dietary TSAA (Table 6). Carcass protein was found to increase quadratically ($Y_{Carcass\ protein}$ =3.5569-17.127X-5.677X^2, R^2=0.993, P<0.05) with the increase in dietary TSAA concentrations. No significant difference in ash content of the fish fed all the test diets was noted. A consistent increasing pattern in the values of carcass fat was recorded at each incremental levels of dietary TSAA (D1-D6). Moisture content showed a reverse pattern to that of carcass fat and it decreased with the increase in dietary TSAA concentrations from D1-D6. The relationship of carcass fat and moisture content to the dietary concentrations of TSAA is depicted by the linear equations which are as follows;

$$Y_{Carcass\ fat}=1.7998+1.682X,\ R^2=0.992;\ P<0.05$$

$$Y_{Moisture\ content}=79.699-3.3908X,\ R^2=0.932;\ P<0.05$$

Somatic indices including HSI, VSI and CF are illustrated in Table 6. Fish fed lowest level of TSAA (D1) showed highest value of HSI and this value decreased significantly as the concentrations of TSAA increased up to 1.31% (D4). The differences in HSI values at higher levels of dietary TSAA (D5-D6) were not significant (P>0.05). The VSI was found to decrease with the increasing concentrations of TSAA until reaching a plateau at 1.31% TSAA (D4). The relationship of VSI to the dietary concentrations of TSAA was described by the quadratic equation (Y_{VSI}=10.732-8.7636X+2.888X^2, R^2=0.987; P<0.05). The CF was found to improve quadratically with increasing levels of dietary TSAA up to 1.31% (D4) but kept relatively constant

thereafter (P>0.05). The quadratic equation for CF was Y_{CF}=-0.1295+2.131X-0.6571X^2, R^2=0.969; P<0.05. A 100% survival of fingerling *C. catla* was recorded under different treatments except those fed diet with lowest level of TSAA where 93% survival was noted (Table 6).

Experiment II: Cystine replacement value for methionine

The influence of diets containing different methionine to cystine ratios on AWG, PER, FE, PG and TSAAG has been depicted in Table 7. These parameters remained almost unchanged (P>0.05) in fish fed diets with methionine to cystine ratio of 80:20 (R1), 70:30 (R2) and 60:40 (R3) and declined significantly (P<0.05) in fish fed diets containing methionine and cystine in a ratio of 50:50 (R4), 40:60 (R5) and 30:70 (R6). Feed intake remained almost identical (P>0.05) in all the treatment groups (Table 7).

Data on carcass composition and somatic indices of fingerling *C. catla* fed diets with different methionine: cystine ratios are presented in Table 8. Carcass protein remained almost same (P>0.05) in fish fed diets with the varying ratios of methionine to cystine up to 60:40 (R3) and, thereafter (R4-R6), reduction in carcass protein was noted. The differences in carcass fat were not statistically distinguishable in fish fed diets with the methionine: cystine ratio of 80:20 (R1) to 60:40 (R3). However, carcass fat increased in fish fed diets R4, R5 and R6. However, moisture content showed a negative trend with the carcass fat. No significant differences in ash content were observed in fish fed all the diets. The CF was not affected (P>0.05) in fish fed diets containing varying ratios of methionine and cystine up to 60:40 (R3). However, a decline in CF in fish fed diets R4 (50:50), R5 (40:60) and R6 (30:70) was noted. The HSI did not show significant changes with the varying ratios of methionine to cystine up to 60:40 (R3). Whereas an increment in HSI in fish fed diets with a methionine and cystine ratios of 50:50 (R4), 40:60 (R5) and 30:70 (R6) was recorded. The VSI remained almost constant (P>0.05) in fish fed diets containing methionine to cystine ratios of 80:20 (R1) to 60:40 (R3) and, thereafter (R4, R5, R6), increased significantly (P<0.05). Per cent survival was not affected by the varying treatment groups (Table 8).

The optimum cystine replacement value for methionine in fingerling *C. catla* was assessed by subjecting AWG, PER, FE, PG and TSAAG to quadratic regression analysis at 95% maximum response. The quadratic regression analysis of AWG (Y_{AWG}=6.311+0.1776X-0.0032X^2, R^2=0.992, P<0.05), PER (Y_{PER}=1.651+0.039X-0.000745X^2, R^2=0.994, P<0.05), FE (Y_{FE}=0.545+0.0128X-0.00024X^2, R^2=0.994, P<0.05), PG (Y_{PG}=1.0222+0.0335X-0.00063X^2, R^2=0.979, P<0.05) and TSAAG (Y_{TSAAG}=23.093+0.7137X-0.0133X^2, R^2=0.986, P<0.05) against varying methionine to cystine ratios exhibited the optimum cystine replacement value for methionine at 39.50, 38.11, 39.07, 37.89 and 39.33%, respectively. The average cystine replacement value for fingerling *C. catla* was found to nearly 39%.

DISCUSSION

High dietary amino acid utilization requires that all amino acids are simultaneously present at adequate concentrations at the sites of protein synthesis. Hence, deficiency of an essential amino acid limits protein synthesis to the level of that particular essential amino acid, the remainder being catabolized (Sveier et al. 2001). Of the various amino acids required for protein synthesis, methionine is clearly the most toxic (Hardwick 1970; Benevenga 1974). Therefore, graded levels of methionine in diets should result in responses for multiple criteria ranging from deficiency through adequacy to excess (Baker 1987). Various statistical approaches have been applied to estimate the methionine requirement of fish (Twibell et al. 2000; Nguyen and Davis 2009; Abidi and Khan 2011). The choice of a statistical model is critical to interpret the nutrient requirement. Generally, non linear models are reckoned as most suitable for evaluating results from dose-response experiments (Cowey 1992; Schutte and Pack 1995; Rodehutscord et al. 1997). Biologically systems rarely work in perfectly linear fashions, so the non-linear models are more intuitive for careful observers of scientific data than models with linear functions (Lerman and Bie 1975). In this study, quadratic regression nalysis of AWG, PER, FE, PG and TSAAG against dietary TSAA concentrations was erformed which yielded the total sulphur amino acid requirement of fingerling *C. catla* : 1.28% dry diet, corresponding to 3.87% of dietary protein which is higher than the :quirement reported for channel catfish (2.34%, Harding et al. 1977); catla (3.55%, Ravi

and Devaraj 1991); carp (3.13%, NRC 2011); rohu (2.52-3.13%, Abidi and Khan 2011); tilapia (3.45%, NRC 2011); but lower than that of mrigal (5.50%, Ahmed et al. 2003); black seabream (5.32% of dietary protein, Zhou et al. 2011b). The wide variations reported in the amino acids requirement studies among species may be due to fish size, age, laboratory condition including feeding regime, feed allowance, water temperature, stock density and ingredients used for experimental diets, such as casein, gelatin, zein, glutin, fishmeal, soyabean meal and crystalline amino acids in various combinations (Kim et al. 1992; Rodehutscord et al. 1997; Luo et al. 2005; Forster and Dominy 2006; Mai et al. 2006a,b). It has also been reported that different levels of cystine and sulfur amino acid-related nutrient such as choline in test diets could also be ascribed to some variability in TSAA requirement (Twibell et al. 2000; Kasper et al. 2000).

Certain dietary EAA deficiencies have been attributed to inferior growth and feed efficiency in most studies (Wilson 2002). In the present study, fish fed diets containing TSAA at sub-optimal levels (D1, D2 and D3) tended to have lower growth. Increasing TSAA concentration in the diets up to 1.31% (D4) improved the growth performance and feed utilization. However, further increase in the dietary TSAA levels (D5-D6) did not show significant differences in growth and feed utilization in this study. The stable growth performance of fish fed diets containing higher levels of TSAA (D5-D6) may be due to the fact that the higher levels of dietary TSAA may not be sufficient to induce toxic effects on the growth of this fish. The stabilized growth performance at higher levels of TSAA was also recorded in earlier studies (Twibell et al. 2000; Nguyen and Davis 2009).

The condition factor is described as an indicator of nutritional status (Love 1970) and the level of reserve nutrients in fish body (Gershanovich et al. 1984). In the present study, gradient increase in dietary TSAA concentrations resulted in a quadratic response in condition factor, which was 1.61 when fed with 1.31% TSAA diet (D4). This increment in CF of fish fed diets containing TSAA up to 1.31% of dry diet (D4) for fingerling *C. catla* possibly indicate that dietary TSAA up to above level increases the nutrient content of the fish body. Especially, the carcass composition analyses indicated an increase of carcass protein in the fish fed diets containing TSAA from 0.56-1.31%

(D1-D4), supporting the result of condition factor. In this study, fish fed TSAA deficient diets (D1, D2 and D3) produced higher values of HSI. Due to low protein gain in TSAA deficient diets, amino acids not used for protein synthesis might have been converted to lipids or glycogen and deposited in the liver, possibly explaining the higher hepatosomatic index in fish receiving diet D1, D2 and D3. The higher values of HSI in fish fed methionine deficient diets have also been recorded in Asian sea bass (Coloso et al. 1999; 2004). Espe et al. (2008) stated that intake of low methionine resulted to higher weight of liver.

The TSAA deficiency resulted in slow growth and reduced feed efficiency and leads to the development of lanticular cataract in several salmonid species (Walton et al. 1982; Keembiyehetty and Gatlin 1993). However, except poor growth and feed utilization efficiency, no cataract was observed in the present study. Absence of cataract due to deficiency of dietary TSAA in this study indicates that the diet containing minimum level of TSAA (0.56%) was sufficient to prevent the cataract in this fish. Poor growth and feed utilization efficiency associated with the deficiency of dietary TSAA were also recorded in earlier studies (NRC 2011; Ahmed 2012b).

Another important aspect of refining a species' requirement for TSAA is determining the cystine sparing value for methionine. Methionine can be converted to cysteine via homocysteine and serine. Presence of cystine in a diet can limit the amount of methionine that must be converted to cystine, thereby reducing the overall amount of methionine required to meet the requirement for TSAA (Lewis 2003). The ability of cystine to spare a portion of the dietary methionine requirement has been demonstrated in various terrestrial animals and several fish species (NRC 2011). In second experiment, growth, feed efficiency and nutrient gain of fish fed diets with different ratios of methionine to cystine up to 60:40 were not affected indicating that cystine could spare 40% of methionine (R3). However, the cystine replacement value for fingerling *C. catla* obtained by quadratic regression analysis was found to be 39% which is lower than the cystine replacement value of channel catfish, 60% (Harding et al. 1977), European sea bass, *D. labrax* 64% (Hidalgo et al. 1987), blue tilapia, *O. aureus* 44% (Liou 1989), rainbow trout, *O. mykiss* 42% (Kim et al. 1992) and red drum, *S. ocellatus* 50% (Goff and

Gatlin III 2004). The present cystine replacement value for methionine is identical to the sparing values of red drum 40% (Moon and Gatlin 1991), hybrid striped bass, *M. saxatilis x M. chrysops* 40% (Griffin et al. 1994) and rohu, 33-39% (Abidi and Khan 2011). There are several factors such as size, water temperature, genetics, feeding rate, energy concentration and other diet factors, and method of data analysis which affect cystine replacement values of fish (Lovell 1989; Abidi and Khan 2011).

When two or more nutrients in a fish diet are interrelated, it is necessary to consider how their presence and quantity will influence the growth of fish and economic benefits of fish production. Methionine is the source of the methyl donor *S*-adenosyl methionine, the metabolite that provides methyl groups in a variety of reactions including the de novo synthesis of choline from phosphatidylethanolamine (El-Gendy et al. 2012). It has been reported that one third of the total methionine supplement can be used to synthesize choline (Emmanuel and Kennelly 1984; Lobley et al. 1996). Because of these metabolic relationships, dietary supply of choline affects methionine requirements and methionine supply can affect choline metabolism (El-Gendy et al. 2012). Since the level of choline in this study was fixed as per the information available on common carp, *C. carpio* (Ogino et al. 1970), there is no probability of interaction between dietary methionine and choline.

Based on above analysis it is recommended that inclusion of 1.28% TSAA, corresponding to 3.87% dietary protein is optimum of which 39% could be spared by cystine. Data generated during the present study would be useful in formulating TSAA balanced, cost-effective feeds for the intensive culture of this fish.

SUMMARY

Two separate 12 weeks feeding trials were performed to quantify the total sulphur amino acid (TSAA) requirement (experiment I) and cystine replacement value for methionine (experiment II) of fingerling *Catla catla*. In experiment I, six casein-gelatin based (33% crude protein; 16.72 kJ/g gross energy) diets with graded levels of TSAA (0.56, 0.81, 1.06, 1.31, 1.56, 1.81% dry diet) were made. The diets were fed to triplicate groups of

fish (3.55±0.06 cm; 0.65±0.02 g) near to satiation. The TSAA requirement was determined by quadratic regression analysis of absolute weight gain (AWG), protein efficiency ratio (PER), feed efficiency (FE), protein gain (PG) and total sulphur amino acid gain (TSAAG) against dietary TSAA concentrations at 95% maximum response. Above analysis revealed that inclusion of TSAA at 1.28% dry diet (1.22% methionine+0.06%cystine) is optimum. In experiment II, to determine the replacement value of cystine for methionine, six diets containing 1.28% TSAA determined in experiment I with different ratios of L-methionine and L-cystine (80:20, 70:30, 60:40, 50:50, 40:60, 30:70) on equimolar sulphur basis were fed to fish (3.65±0.08 cm; 0.67±0.04 g). Quadratic regression analysis of AWG, PER, FE, PG and TSAAG against varying methionine to cystine ratios yielded the optimum cystine replacement value of about 39%. Based on above analysis, it is recommended that inclusion of 1.28% dietary TSAA, corresponding to 3.87% of dietary protein is optimum of which 39% could be spared by cystine. Data generated during this study would be useful in formulating TSAA balanced, cost-effective feeds for the intensive culture of this fish.

Table 1 Composition of the basal diet (D1) used in experiment I

Ingredients	Basal diet (%)
Casein[a] (vitamin and fat-free)	14
Gelatin[b]	4.67
Dextrin	34.38
Amino acid mixture[c]	18.16
Corn oil	5
Cod liver oil	2
Mineral mix[d,f]	4
Vitamin mix[e,f]	3
α- Cellulose	4.79
Carboxymethyl cellulose	10
Total	100
Analyzed crude protein (% dry diet)	32.89
Calculated gross energy (kJ/g, dry diet)	16.72

[a]Crude Protein (76%); [b]Crude Protein (96%); [c]Amino acid mixture (% dry diet) arginine 1.227, histidine 0.291, isoleucine 1.807, leucine 1.543, lysine 1.043, methionine 0, cystine 0, phenylalanine 1.295, tyrosine 0.762, threonine 0.851, tryptophan 0.383, valine 1.385, alanine 1.004, proline 0.678, glycine 6.535 (Loba Chemie, India); [d,f]Mineral mixture (g/100g of mineral mix) calcium biphosphate 13.57; calcium lactate 32.69; ferric citrate 02.97; magnesium sulphate 13.20; potassium phosphate (dibasic) 23.98; sodium biphosphate 08.72; sodium chloride 04.35; aluminium chloride. $6H_2O$ 0.0154; potassium iodide 0.015; cuprous chloride 0.010; manganous sulphate. H_2O 0.080; cobalt chloride. $6H_2O$ 0.100; zinc sulphate. $7H_2O$ 0.40; [e,f]Vitamin mixture (g/100g dry diet) choline chloride 0.400; inositol 0.200; ascorbic acid 0.100; niacin 0.075; calcium pantothenate 0.05; riboflavin 0.02; menadione 0.004; pyridoxine hydrochloride 0.005; thiamin hydrochloride 0.005; folic acid 0.0015; biotin 0.0005; alpha-tocopherol 0.04; vitamin B_{12} 0.00001; 2.1 g α-cellulose; [f]Halver (2002).

Table 2 Analyzed amino acid composition of the basal diet (D1) used in experiment I[a]

Amino acid	Basal diet (%)
EAAs	
Arginine	2.13
Histidine	0.71
Isoleucine	2.67
Leucine	3.06
Lysine	2.37
Methionine	0.50
Phenylalanine	2.04
Threonine	1.44
Tryptophan	0.53
Valine	2.39
NEAAs	
Cystine	0.05
Tyrosine	1.50
Alanine	1.91
Aspartic acid	1.15
Glycine	7.47
Proline	2.73
Serine	0.59

[a]Determined by Hitachi L-8800 Automatic Amino Acid Analyzer

Table 3 *Composition of the experimental diet (R1) used in experiment II*

Ingredients	Experimental diet (%)
Casein[a] (vitamin and fat-free)	9.55
Gelatin[b]	3.18
Dextrin	34.32
Amino acid mixture[c]	23.66
Corn oil	5
Cod liver oil	2
Mineral mix[d,f]	4
Vitamin mix[e,f]	3
α- Cellulose	5.29
Carboxymethyl cellulose	10
Total	100
Analyzed crude protein (% dry diet)	32.81
Calculated gross energy (kJ/g, dry diet)	16.72

[a]Crude Protein (76%); [b]Crude Protein (96%); [c]Amino acid mixture (% dry diet) arginine 1.509, histidine 0.419, isoleucine 2.072, leucine 2.017, lysine 1.467, methionine 0.640, cystine 0.262, phenylalanine 1.544, tyrosine 0.992, threonine 1.034, tryptophan 0.419, valine 1.710, alanine 1.289, aspartic acid 0.358, glutamic acid 0.011, glycine 6.483, proline 1.345, serine 0.093 (Loba Chemie, India). [d,f]Mineral mixture (g/100g of mineral mix) calcium biphosphate 13.57; calcium lactate 32.69; ferric citrate 02.97; magnesium sulphate 13.20; potassium phosphate (dibasic) 23.98; sodium biphosphate 08.72; sodium chloride 04.35; aluminium chloride. $6H_2O$ 0.0154; potassium iodide 0.015; cuprous chloride 0.010; manganous sulphate. H_2O 0.080; cobalt chloride. $6H_2O$ 0.100; zinc sulphate. $7H_2O$ 0.40; [e,f]Vitamin mixture (g/100g dry diet) choline chloride 0.400; inositol 0.200; ascorbic acid 0.100; niacin 0.075; calcium pantothenate 0.05; riboflavin 0.02; menadione 0.004; pyridoxine hydrochloride 0.005; thiamin hydrochloride 0.005; folic acid 0.0015; biotin 0.0005; alpha-tocopherol 0.04; vitamin B_{12} 0.00001; 2.1 g α-cellulose; [f]Halver (2002).

Table 4 Analyzed amino acid composition of the experimental diet (R1) used in experiment II[a]

Amino acid	Experimental diet (%)
EAAs	
Arginine	2.11
Histidine	0.69
Isoleucine	2.64
Leucine	3.09
Lysine	2.34
Methionine	0.98
Phenylalanine	2.06
Threonine	1.45
Tryptophan	0.52
Valine	2.41
NEAAs	
Cystine	0.29
Tyrosine	1.48
Alanine	1.90
Aspartic acid	1.15
Glycine	7.46
Proline	2.71
Serine	0.60

[a]Determined by Hitachi L-8800 Automatic Amino Acid Analyzer

Table 5 Growth performance of fingerling *C. catla* of fish fed diets containing varying levels of total sulphur amino acid[a,b]

	Total sulphur amino acid levels (% dry diet)					
	0.56 (D1)	0.81 (D2)	1.06 (D3)	1.31 (D4)	1.56 (D5)	1.81 (D6)
Average initial weight (g)	0.65±0.02[a]	0.65±0.02[a]	0.66±0.02[a]	0.65±0.04[a]	0.65±0.06[a]	0.65±0.02[a]
Average final weight (g)	3.74±0.08[d]	6.04±0.08[c]	7.77±0.05[b]	9.60±0.11[a]	9.55±0.08[a]	9.41±0.11[a]
Absolute weight gain (g/fish)	3.09±0.04[d]	5.39±0.06[c]	7.11±0.08[b]	8.95±0.13[a]	8.90±0.12[a]	8.76±0.09[a]
Feed efficiency	0.25±0.02[d]	0.43±0.02[c]	0.56±0.01[b]	0.71±0.02[a]	0.70±0.02[a]	0.69±0.01[a]
Feed intake (g/fish)	12.36±0.13[a]	12.53±0.15[a]	12.69±0.14[a]	12.61±0.16[a]	12.70±0.13[a]	12.69±0.15[a]
Protein efficiency ratio	0.76±0.02[d]	1.30±0.03[c]	1.70±0.02[b]	2.15±0.05[a]	2.12±0.07[a]	2.09±0.03[a]
Protein gain (g/fish)	0.34±0.02[d]	0.75±0.01[c]	1.10±0.02[b]	1.49±0.02[a]	1.46±0.021[a]	1.43±0.032[a]
TSAAG (mg/fish)[c]	13.87±0.12[d]	22.09±0.23[c]	30.46±0.37[b]	35.58±0.42[a]	34.95±0.41[a]	34.36±0.38[a]

[a]Mean values of 3 replicates±SEM; [b]Mean values sharing the same superscripts in the same row are insignificantly different (P>0.05); [c]Total sulphur amino acid gain

Table 6 Carcass composition (%wet basis) and somatic indices of fingerling *C. catla* of fish fed diets containing varying levels of total sulphur amino acid[a,b]

	Total sulphur amino acid levels (% dry diet)						
	Initial	0.56 (D1)	0.81 (D2)	1.06 (D3)	1.31 (D4)	1.56 (D5)	1.81 (D6)
Moisture (%)	77.14±0.94	77.35±0.91^{a}	77.61±0.81^{a}	76.12±1.15^{ab}	75.18±1.08^{b}	74.15±1.03^{c}	73.68±1.09^{c}
Protein (%)	12.81±0.13	11.29±0.11^{d}	13.86±0.19^{c}	15.23±0.16^{b}	16.42±0.19^{a}	16.21±0.14^{a}	16.07±0.21^{a}
Fat (%)	3.05± 0.09	2.81± 0.02^{f}	3.19± 0.04^{e}	3.48± 0.08^{d}	3.92± 0.05^{c}	4.45±0.04^{b}	4.91± 0.06^{a}
Ash (%)	2.11± 0.05	2.14±0.03^{a}	2.16±0.02^{a}	2.13± 0.01^{a}	2.15±0.04^{a}	2.14±0.02^{a}	2.11± 0.03^{a}
Hepatosomatic index%	-	1.09±0.04^{a}	0.91±0.02^{b}	0.82±0.03^{c}	0.74±0.02^{d}	0.73±0.04^{d}	0.74±0.02^{d}
Viscerosomatic index%	-	6.72±0.05^{a}	5.63±0.03^{b}	4.52±0.04^{c}	4.21±0.04^{d}	4.24±0.06^{d}	4.26±0.02^{d}
Condition factor	-	0.86±0.02^{d}	1.19±0.02^{c}	1.31±0.04^{b}	1.61±0.02^{a}	1.58±0.03^{a}	1.57±0.04^{a}
Survival%	-	93	100	100	100	100	100

[a]Mean values of 3 replicates±SEM; [b]Mean values sharing the same superscripts in the same row are insignificantly different (P>0.05).

Table 7 Growth performance of fingerling *Catla catla* fed diets containing varying methionine to cystine ratios[a,b]

	Dietary methionine to cystine ratios					
	80:20 (R1)	70:30 (R2)	60:40 (R3)	50:50 (R4)	40:60 (R5)	30:70 (R6)
Initial weight (g/fish)	0.67±0.02[a]	0.67±0.05[a]	0.67±0.02[a]	0.66±0.03[a]	0.67±0.07[a]	0.67±0.08[a]
Final weight (g/fish)	9.24±0.14[a]	9.28±0.12[a]	9.31±0.11[a]	7.67±0.07[b]	5.90±0.09[c]	3.79±0.06[d]
Absolute weight gain (g/fish)	8.57±0.02[a]	8.61±0.14[a]	8.64±0.11[a]	7.01±0.17[b]	5.23±0.02[c]	3.12±0.05[d]
Feed efficiency	0.70±0.03[a]	0.71±0.05[a]	0.69±0.02[a]	0.56±0.02[b]	0.42±0.04[c]	0.25±0.02[d]
Feed intake (g/fish)	12.24±0.17[a]	12.13±0.13[a]	12.52±0.12[a]	12.51±0.12[a]	12.45±0.15[a]	12.48±0.12[a]
Protein efficiency ratio	2.12±0.02[a]	2.15±0.03[a]	2.09±0.04[a]	1.70±0.02[b]	1.27±0.02[c]	0.76±0.01[d]
Protein gain (g/fish)	1.42±0.03[a]	1.44±0.05[a]	1.45±0.03[a]	1.08±0.02[b]	0.65±0.01[c]	0.31±0.02[d]
TSAAG (mg/fish)[c]	31.89±0.21[a]	32.03±0.19[a]	32.15±0.23[a]	25.31±0.18[b]	16.53±0.16[c]	8.93±0.14[d]

[a]Mean values of 3 replicates±SEM; [b]Mean values sharing the same superscripts in the same row are insignificantly different (P>0.05); [c]Total sulphur amino acid gain

Table 8 Carcass composition (%wet basis) and somatic indices of fingerling *Catla catla* fed diets containing varying methionine to cystine ratios[a,b]

	Dietary methionine to cystine ratios						
	Initial	80:20 (R1)	70:30 (R2)	60:40 (R3)	50:50 (R4)	40:60 (R5)	30:70 (R6)
Moisture (%)	79.02±0.74	77.11±0.81[a]	77.14±0.95[a]	77.12±0.88[a]	76.42±0.93[ab]	75.53±0.89[b]	74.26± 0.74[c]
Protein (%)	12.67±0.14	16.31±0.15[a]	16.42±0.19[a]	16.46±0.21[a]	15.14±0.14[b]	14.09±0.18[c]	12.65±0.16[d]
Fat (%)	3.28± 0.11	3.11± 0.02[d]	3.12± 0.08[d]	3.15±0.05[d]	3.48±0.06[c]	3.92±0.02[b]	4.63±0.04[a]
Ash (%)	2.13± 0.04	2.15±0.05[a]	2.16± 0.01[a]	2.11±0.04[a]	2.17±0.02[a]	2.21± 0.03[a]	2.18± 0.02[a]
Hepatosomatic index%	-	0.89±0.05[c]	0.91±0.04[c]	0.90±0.03[c]	0.97±0.04[b]	1.07±0.02[ab]	1.15±0.02[a]
Viscerosomatic index%	-	4.15±0.07[d]	4.18±0.05[d]	4.16±0.04[d]	4.63±0.05[c]	5.32±0.03[b]	5.91±0.03[a]
Condition factor	-	1.57±0.02[a]	1.61±0.04[a]	1.62±0.02[a]	1.51±0.02[b]	1.38±0.03[c]	1.21±0.04[d]

[a]Mean values of 3 replicates±SEM; [b]Mean values sharing the same superscripts in the same row are insignificantly different (P>0.05).

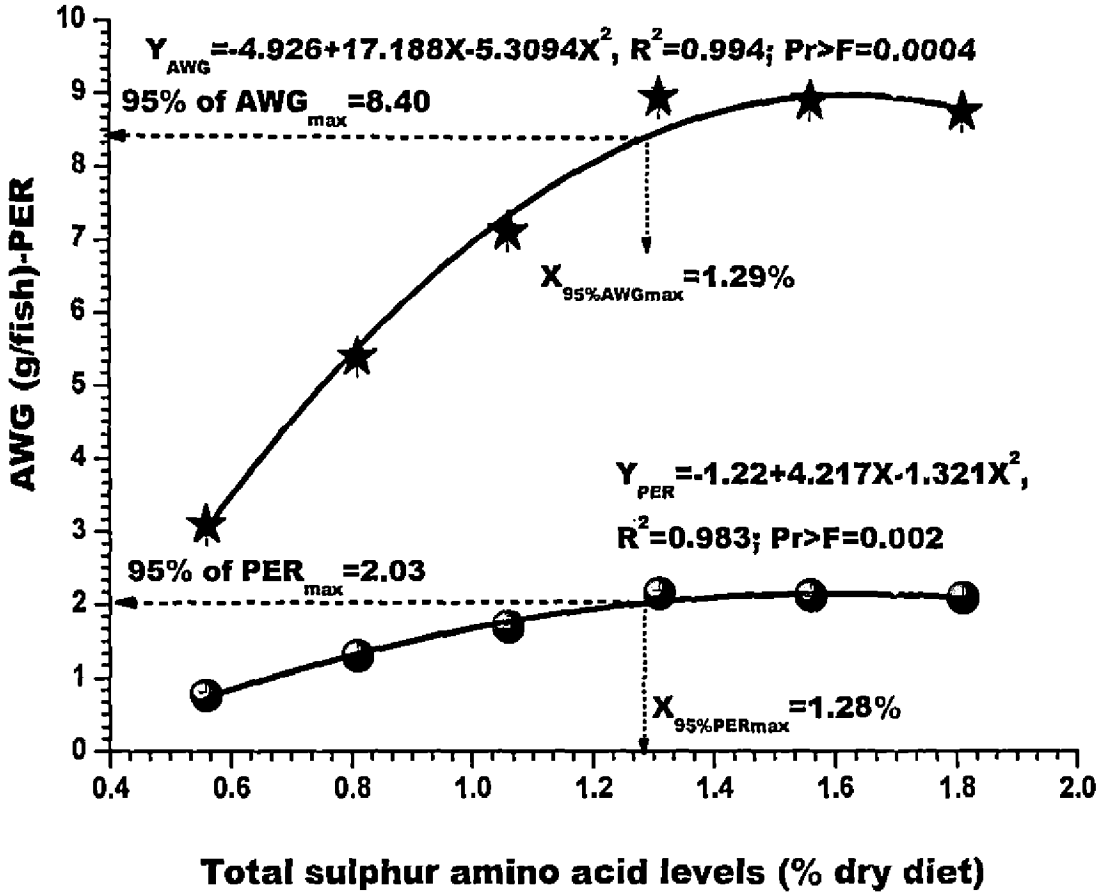

Fig. 1 Quadratic relationship of dietary total sulphur amino acid to absolute weight gain and protein efficiency ratio

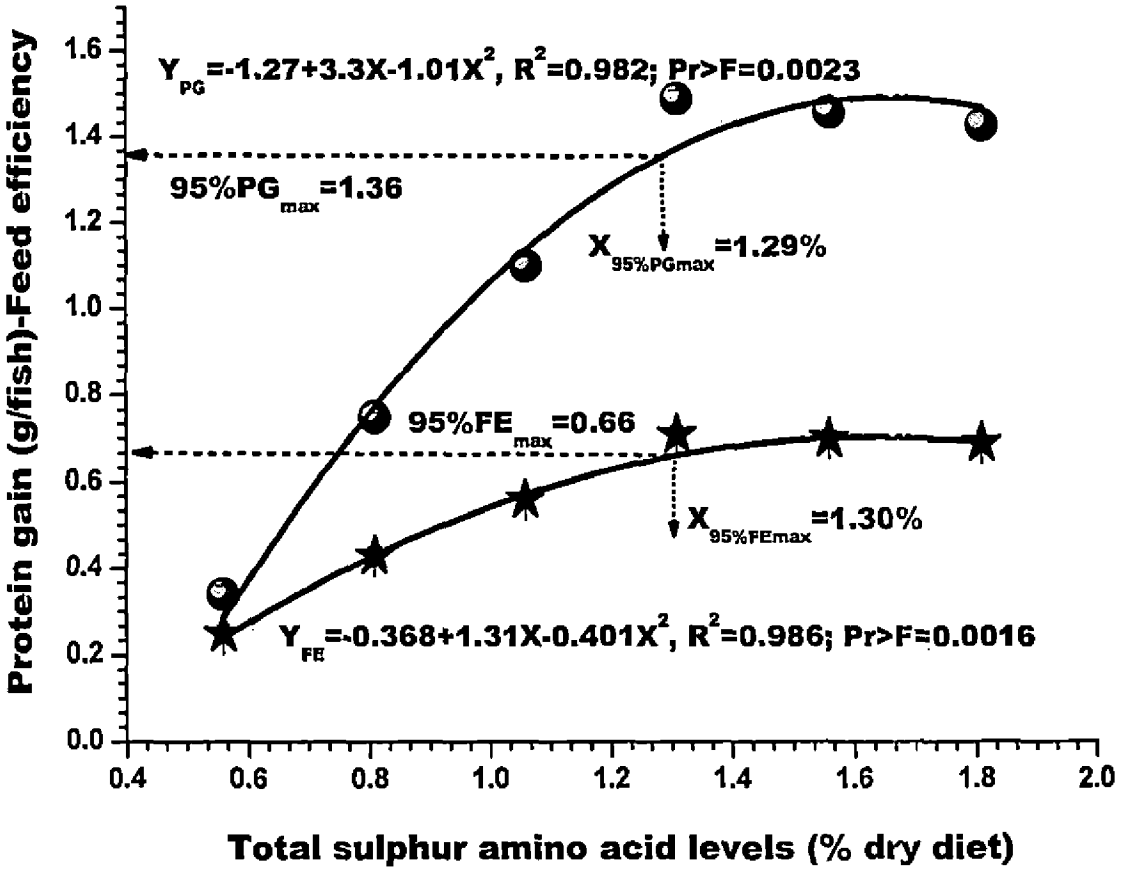

Fig. 2 Quadratic relationship of dietary total sulphur amino acid to protein gain and feed efficiency

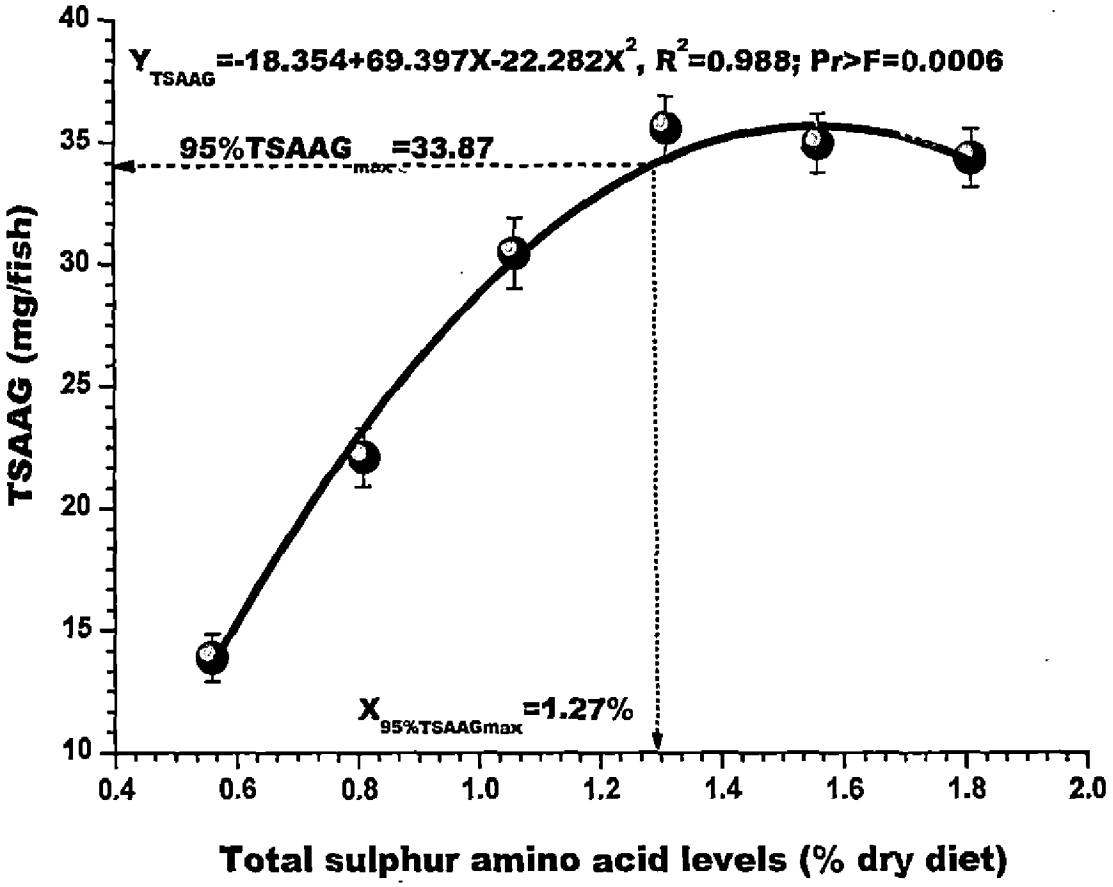

Fig. 3 Quadratic relationship of dietary total sulphur amino acid to total sulphur amino acid gain

CHAPTER 10

CHAPTER 10

DIETARY PHENYLALANINE REQUIREMENT AND TYROSINE REPLACEMENT VALUE FOR PHENYLALANINE FOR FINGERLING *CATLA CATLA* (HAMILTON)

INTRODUCTION

Feed is one of the major inputs in aquaculture, and the success of fish farming depends primarily on the provision of adequate quantity of nutritionally balanced feeds in a form, which is acceptable to fish (Zargar et al. 2012). Hence, the inclusion of optimum quantity of a particular nutrient is necessary for successful aquaculture system. Determining the essential amino acid requirements of cultured fish is extremely important because of significant effects of these nutrients on muscle deposition, feed cost, and nitrogen pollution (Small and Soares 1999). They are important fuel molecules, signalling factors and major substrates for the synthesis of a wide range of bioactive molecules and proteins (Finn and Fyhn 2010). Being the building blocks of protein synthesis, amino acids in fish are also used in energy production or for other metabolic purposes (Ronnestad et al. 2001). Quantitative dietary requirements for the ten indispensable amino acids have been determined for several fish species (Wilson 2002; NRC 2011).

Phenylalanine, an aromatic indispensable amino acid is required for normal growth and metabolic processes. It is the sole precursor of tyrosine. Phenylalanine can be converted to tyrosine by tetrahydrobiopterin-dependent phenylalanine hydroxylase in liver and kidneys but phenylalanine can not be synthesized back from tyrosine (Benakappa and Varghese 2004; Li et al. 2009; Kim et al. 2012). Thus, adding tyrosine to diets for fish can reduce requirement for phenylalanine. Tyrosine is a common precursor for important hormones and neurotransmitters, including thyroxine (T4), triiodothyronine, epinephrine, nor-epinephrine, dopamine, and melanin (Li et al. 2009). Pinto et al. (2009) reported that dietary requirements for phenylalanine and tyrosine of fish increase substantially during metamorphism. These molecules have important regulatory roles (Chang et al. 2007; Yoo et al. 2000). Information on the effects of

phenylalanine and tyrosine on growth is scarce. Hence, inclusion of sufficient amounts of phenylalanine and tyrosine to optimize the growth, body protein synthesis and also for the other physiological functions in fish is essential.

Dietary total aromatic amino acid requirements (TAAA) have been ascertained for a number of fish species (NRC 2011). Dietary phenylalanine requirement of fry *C. catla* has been reported (Ravi and Devaraj 1991). No information on TAAA requirement and tyrosine replacement value for phenylalanine for the fingerling stage of this fish is available. Therefore, this study was aimed to investigate the dietary TAAA requirement and tyrosine replacement value for phenylalanine for fingerling *C. catla*.

MATERIALS AND METHODS

Experimental diets

Two experiments were conducted to determine the total aromatic amino acid requirement and the tyrosine replacement value for phenylalanine in fingerling *C. catla*. For conducting experiment I, six isonitrogenous (33% crude protein) and isocaloric (16.72 kJ/g gross energy) amino acid test diets (D1, D2, D3, D4, D5 and D6) using casein (fat-free), gelatin and crystalline L-amino acids with graded levels of phenylalanine (0.40, 0.65, 0.90, 1.15, 1.40 and 1.65% dry diet) with fixed level (1%) of tyrosine were prepared. The level of tyrosine in the amino acid test diets was fixed on the basis of information available on other Indian major carp namely *Cirrhinus mrigala* (Ahmed 2009). The amount of phenylalanine contributed by casein and gelatin in basal diet (D1) was 0.36 and 0.04%, respectively. Incremental levels of L-phenylalanine (0.25, 0.5, 0.75, 1.0 and 1.25%) were added to prepare D2, D3, D4, D5 and D6 diets. The analyzed values of phenylalanine in experimental diets were found to be 0.39, 0.64, 0.87, 1.12, 1.38 and 1.62% dry diet. The levels of phenylalanine in the amino acid test diets were fixed on the basis of information available on other carps (Khan and Abidi 2007b; Ahmed 2009; NRC 2011). In Experiment II, six diets with different levels of L-tyrosine (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2% dry diet) with fixed phenylalanine (1.01% dry diet) were formulated to determine the tyrosine requirement. The level of phenylalanine in all the experimental

diets was fixed as per the requirement determined in experiment I. The diets were marked as T1, T2, T3, T4, T5 and T6. The amount of tyrosine contributed by casein and gelatin in basal diet (T1) was 0.15 and 0.05%, respectively. Incremental levels of crystalline L-tyrosine (0.2, 0.4, 0.6, 0.8 and 1.0%) were added to prepare T2, T3, T4, T5 and T6 diets. The analyzed values of tyrosine in experimental diets were found to be 0.19, 0.38, 0.59, 0.81, 0.98 and 1.18% dry diet. Levels of tyrosine in the amino acid test diets were fixed on the basis of information available on other fish species (Kim 1993; Ahmed 2009; NRC 2011). The compositions of the basal diets used in experiment I and II are given in Table 1. The analyzed amino acid compositions of the basal diets used in above experiments are presented in Table 2. The diets were made isonitrogenous by adjusting the level of glycine. Crystalline L-amino acids, excluding the phenylalanine and tyrosine, were used to simulate the amino acid profile of the experimental diets to that of 33% whole chicken egg protein. Method of preparation of experimental diets has been discussed under General Methodology section (pages 9-10).

Experimental design and feeding trials

Source of the fish, their acclimation and details of the general experimental design has already been discussed under the General Methodology section (page 8).

For conducting experiment I, fingerling *C. catla* (3.95±0.24 cm; 0.68±0.19 g) were taken from the above acclimated fish lot and stocked in triplicate groups in 70 L circular polyvinyl troughs (water volume 55 L) fitted with a continuous water flow-through (1-1.5 L/min) system at the rate of 25 fish per trough for each dietary treatment level. Fish were fed test diets in the form of dry crumbles (500 µm) to apparent satiation thrice a day at 08:00, 12:30 and 17:30h. Initial and weekly weights were recorded on a top-loading balance (Precisa 120A; 0.1 mg sensitivity, Oerlikon AG, Zurich, Switzerland) after anaesthetizing the fish with tricaine methane sulfonate (MS-222; 100 µg/ml). Fish were deprived of feed on the day they were weighed. The feeding trials lasted for 12 weeks. Faecal matter was siphoned before every feeding. For conducting experiment II, fingerling *C. catla* of almost similar size (3.85±0.25 cm; 0.66±0.16 g) were selected. Feeding trial was conducted following the same procedures as used in

experiment I. Water quality indices were monitored daily during the feeding trial and recorded following standard methods (APHA 1992). The range of water temperature, dissolved oxygen, free carbon dioxide, pH, total ammonia nitrogen, nitrites and total alkalinity based on daily measurements, was 26.5-27.9°C, 6.7-7.5 mg/L, 6.2-9.8 mg/L, 7.1-7.8, 0.28-0.31 mg/L, 0.04-0.09 mg/L and 68.5-79.9 mg/L, respectively.

Chemical analyses

Proximate composition of casein, gelatin, experimental diets, and initial and final carcass was estimated using standard methods (pages 10-11). Gross energy content was determined on a Gallenkamp Ballistic Bomb Calorimeter as per the method described on page 12. Amino acid analysis of casein, gelatin, experimental diets, initial and final fish carcass was done using an automatic amino acid analyzer as detailed earlier (page 12). At the beginning of the feeding trial, 60 fish were randomly sampled, killed and pooled together. Six subsamples of a pooled sample were analyzed for initial carcass composition. At the end of the experiment, 20 fish from each replicate of dietary treatments were randomly collected, sacrificed with an overdose of the MS-222 and pooled separately. Three subsamples of the pooled samples were analyzed for final carcass composition.

Determination of RNA and DNA

RNA and DNA were determined by the method of Schneider (1957) as detailed earlier on page 13.

Evaluation of growth parameters

Calculation of various growth parameters was made according to the standard definitions as described under the General Methodology section (pages 13-14).

Statistical analyses

Statistical analyses of growth data were done using procedures as described earlier (page 14).

RESULTS

Experiment 1: Phenylalanine requirement

Use of several response parameters such as absolute weight gain (AWG, g/fish), feed conversion ratio (FCR), protein retention efficiency (PRE%), phenylalanine retention efficiency (PHRE%) and RNA/DNA ratio may furnish the precise phenylalanine requirement than weight gain data alone. These parameters improved with the increasing concentrations of dietary phenylalanine and peaked (AWG 8.23 g/fish; FCR 1.47; PRE 35.99%; PHRE 76.53% and RNA/DNA ratio 5.48) at 1.12% phenylalanine of the dry diet (D4). Further inclusion of dietary phenylalanine (D5-D6) resulted in reduction in these parameters. Feed intake remained almost same ($P>0.05$) among the varying dietary treatment groups (Table 3). The dietary phenylalanine requirement of fingerling *C. catla* was determined by subjecting above growth data to quadratic regression analysis at 95% of maximum response. The regression analysis of AWG (Fig. 1), PRE (Fig. 1), PHRE (Fig. 2) and RNA/DNA ratio (Fig. 3) against dietary phenylalanine concentrations yielded the requirement at 1.02, 0.99, 0.97 and 1.06% of dry diet, respectively.

Dietary inclusion of phenylalanine had significant ($P<0.05$) impact on carcass composition of fingerling *C. catla* (Table 4). Carcass protein increased with the increasing levels of dietary phenylalanine reaching the highest value at 1.12% (D4) and was found to decline ($P<0.05$) thereafter (D5-D6). A continuous increment in the carcass fat was noted in fish fed diets D1 to D6. However, moisture content was found to correlate negatively with the increasing concentrations of dietary phenylalanine. Ash content remained constant in all the experimental groups. A 100% survival of fingerling *C. catla* was noted among varying treatments except those fed diet with lowest level of phenylalanine (D1) where 94% survival was recorded (Table 4).

Experiment 2: Tyrosine requirement

The effect of varying levels of tyrosine on AWG, FCR, PRE, PHRE and RNA/DNA ratio are presented in Table 5. Fish fed diets containing 0.2% (T1), 0.4% (T2), 0.6% (T3) and 0.8% (T4) dietary tyrosine showed gradual and significant ($P<0.05$) improvements in

AWG, FCR, PRE, PHRE and RNA/DNA ratio. However, further increment in tyrosine concentrations at 1.0% (T5) and 1.2% (T6) of dry diet depressed growth in terms of above parameters. Feed intake was not affected among all the treatment groups. Tyrosine requirement of fingerling *C. catla* was evaluated by using quadratic regression analysis at 95% maximum response. The regression analysis of AWG (Fig. 4), PRE (Fig. 4), PHRE (Fig. 5) and RNA/DNA ratio (Fig. 6) against the varying levels of dietary tyrosine reflected the requirement at 0.64, 0.65, 0.71 and 0.73% dry diet, respectively.

Carcass composition of fish fed varying levels of tyrosine is depicted in Table 6. Carcass protein increased markedly with the increasing concentrations of tyrosine up to 0.8% diet (T4). However, further increment in tyrosine levels (T5-T6) resulted to a significant decline in carcass protein. Carcass fat increased significantly ($P<0.05$) in fish fed diets with the increasing levels of tyrosine up to 0.8% (T4) and then leveled off in fish fed T5 and T6 diets. However, moisture content showed a reverse trend to the carcass fat. No significant differences ($P>0.05$) in ash content were noted in fish fed diets with variable levels of tyrosine. A 100% survival was recorded in all the dietary treatment groups.

Based on above results, total aromatic amino acid requirement was found to be 1.69% of diet (1.01% phenylalanine+0.68% tyrosine), corresponding to 5.12% of dietary protein. The tyrosine replacement value for phenylalanine was found to be 40.2% (0.68/1.01+0.68 x 100) on a weight and 36.7% (165.19/181.19 x 40.2) on a molar basis.

DISCUSSION

Fish have a total aromatic amino acid requirement rather than a specific phenylalanine requirement. Thus, inclusion of an optimum amount of total aromatic amino acid is a prerequisite to the formulation of nutritionally adequate artificial feeds for the culture of this fish. The present finding indicates that inclusion of 1.69% (1.01% phenylalanine+0.68% tyrosine) total aromatic amino acids of the dry diet, corresponding to 5.12% of dietary protein, is optimum for maximizing growth of fingerling *C. catla*. The TAAA requirement (5.12% of dietary protein) determined for fingerling *C. catla* in

this study is lower than that of common carp *Cyprinus carpio* 5.97% (Ogino 1980); rohu *Labeo rohita* 5.53% (Murthy and Varghese 1997d); silver perch *Bidyanus bidyanus* 5.75% (Ngamsnae et al. 1999); rohu, 5.4-5.55% (Khan and Abidi 2007b); mrigal *Cirrhinus mrigala* 5.38% (Ahmed 2009); gibel carp *Carassius gibelio* 5.61% (Ying et al. 2010); but higher than that reported for rainbow trout *Oncorhynchus mykiss* 4.3% (Kim 1993) and approximately equal to the requirement of channel catfish *Ictalurus punctatus* 5.0% (Robinson et al. 1980) of dietary protein.

The wide differences in amino acid requirements may be due to the differences in the methodologies used such as experimental conditions, feeding regime, feed allowance, water temperature, stock density, ingredient combinations of dietary protein sources used for basal diets such as casein, gelatin, crystalline amino acid in varying combinations, adequate levels of other nutrients and also due to the use of different variables and statistical analyses to establish the requirements (Kim et al. 1992; Akiyama et al. 1997; Ruchimat et al. 1997; Forster and Ogata 1998; Ahmed 2009). Digestibility, amino acid profile, and energy content may also contribute to the discrepancies among the amino acid requirements (Simmons et al. 1999; De Silva et al. 2000). Selection of response criteria is another important variable that may influence the estimate of the requirements (Rodehutscord et al. 1997).

Fish fed diets containing deficient amount of phenylalanine (D1, D2, D3) caused growth retardation in fingerling *C. catla* in this study. Weight gain was found to increase as dietary phenylalanine increased to a level of 1.12% (D4) and further inclusion of dietary phenylalanine (D5 and D6) led to reduction in weight gain. This declining trend in weight gain at higher levels of phenylalanine may be attributed to toxic effects (Choo et al. 1991) and the use of energy in nitrogenous excretion because excess amino acid is deaminated and excreted in the form of ammonia (Walton 1985). The growth reduction in fish fed diets containing surplus of phenylalanine could also be a consequence of dietary amino acid imbalance. The reduction in growth of fish fed diets containing higher levels of phenylalanine has also been reported for milk fish *Chanos chanos* (Borlongan 1992); rohu *L. rohita* (Murthy and Varghese 1997d; Khan and Abidi 2007b) and mrigal *C. mrigala* (Ahmed 2009).

Growth rate is directly linked to protein synthesis, which in turn is related to the amount of RNA in cells. Since the amount of DNA in cell remains relatively constant, the RNA/DNA ratio would provide a good indication of the rate of protein synthesis and hence growth (Mustafa 1977; 1978; 1979; 1983; Mustafa and Jafri 1977; Mustafa and Mittal 1982; Mustafa and Zofair 1985; Mustafa et al. 1991; Zhou et al. 2001; Zehra and Khan 2013a,b,c). Indeed, RNA/DNA ratio has been considered a promising indicator of growth in response to dietary amino acids in fish species (Di et al. 2009; Abidi and Khan 2009; Zehra and Khan 2013a,b,c). Improvement in RNA/DNA ratio with the incremental levels of dietary phenylalanine up to 1.12% indicated that the fish fed diet D4 were more actively synthesizing and accumulating protein, resulting in better growth performance than fish with low RNA/DNA ratio receiving either low or high phenylalanine diets.

Carcass composition of fingerling *C. catla* was affected by the varying concentrations of dietary phenylalanine. Carcass fat showed the positive trend with the increasing concentrations of dietary phenylalanine. Higher level of phenylalanine was probably deaminated and provided the carbon skeletons for lipid synthesis by the process of lipogenesis which is evident by high amount of carcass lipid in fish fed diets D5 and D6 (Table 4). In present study, protein retention and carcass protein were found to be positively correlated to phenylalanine concentrations up to 1.12% of the dry diet. Maximum protein retention at this level of phenylalanine indicates more efficient utilization of phenylalanine for body protein synthesis.

Amino acid retention is an important parameter in determining the amino acid requirements of fish (Peres and Oliva-Teles 2008; Farhat and Khan 2013b; Zehra and Khan 2013a,b,c). Thus, in this study, phenylalanine retention efficiency has also been considered to estimate the phenylalanine requirement of fingerling *C. catla*. Phenylalanine retention efficiency showed a consistent increasing response to increasing levels of phenylalanine up to 1.12% of the dry diet. Maximum phenylalanine retention at above level of phenylalanine may be due to the best utilization of phenylalanine for protein synthesis.

In experiment II of this study, fish fed diet with adequate tyrosine (0.8%) with

optimum phenylalanine (1.01%) reflected highest gain in weight which declined thereafter indicating that the excess tyrosine (1-1.2%) in fish fed diets T5 and T6 might have adversely affected the growth. It has been reported that excess levels of dietary tyrosine may be toxic in certain animals (Harper et al. 1970). A similar toxic effect of excess dietary tyrosine as evident in this study has also been reported in *C. mrigala* (Ahmed 2009). Contrary to this, Robinson et al. (1980) have reported that the fingerling channel catfish is not sensitive to large excesses of dietary tyrosine.

Tyrosine is reported to be dispensable for growth in fish (Nose et al. 1974). Tyrosine is the precursor of dopamines and the adrenocortical hormones nor-epinephrine (NE) and adrenaline. Dopamines regulate central and peripheric nervous system activity and can, therefore, be related to the control of stress in the fish (Lehnert and Wurtman 1993). Tyrosine has been shown to reduce acute stress such as cold exposure in rodents when supplemented in the diet (Brady et al. 1980; Lehnert et al. 1984). In the present study, the tyrosine requirement estimated using growth data was found to be 0.8% of dry diet, corresponding to 2.42% of dietary protein with fixed level (1.01%) of phenylalanine. The results from this study showed that catla fingerling fed at lower levels of tyrosine (T1-T3) retained significantly less phenylalanine than that of the groups fed on 0.8% (T4) tyrosine diet (Table 5). The reduced retention of phenylalanine in fish fed diets containing lower levels of dietary tyrosine (T1-T3) may be because of the fact that phenylalanine gets converted to tyrosine. Highest phenylalanine retention efficiency in fish fed diet containing 0.8% tyrosine (T4) indicates that inclusion of tyrosine at this level is adequate and prevents the conversion of phenylalanine to tyrosine. Thus, inclusion of the dispensable amino acid tyrosine in the diet induces a saving effect for phenylalanine by sparing the conversion of latter into the former. Since adequate amount of dietary tyrosine spares phenylalanine, it is essential to work out the proper ratio of phenylalanine and tyrosine for optimum growth of fish. The ability of tyrosine to spare a portion of the dietary phenylalanine requirement has been demonstrated in various terrestrial animals and several fish species (NRC 2011). The replacement value of tyrosine for phenylalanine for fingerling *C. catla* was found to be 40.2% on a weight basis or 36.7% on a molar basis. Above tyrosine replacement value for phenylalanine is comparable to

the replacement value reported for Nile tilapia *Oreochromis niloticus* (40% on a weight basis; Santiago and Lovell 1988); mrigal *C. mrigala* (39.53% on a weight and 36% on a molar basis; Ahmed 2009); and is lower than the value reported for common carp *C. carpio* (60% on a weight basis; Nose 1979); channel catfish *I. punctatus* (50% on a molar basis; Robinson et al. 1980); milkfish *C. chanos* (46% on a weight basis; Borlongan 1992) and rainbow trout *O. mykiss* (53% on a weight and 48% on a molar basis; Kim 1993).

The conversion of phenylalanine to tyrosine complicates the estimation of a precise phenylalanine requirement as the level of dietary tyrosine will vary the amount of dietary phenylalanine required by the fish (NRC 2011). Nose (1979) reported that phenylalanine requirement of common carp was 6.5% of dietary protein with the absence of tyrosine, but reduced by up to 3.4% of dietary protein when 1.0% tyrosine was included in the diet. Nose (1979) also reported similar results for Japanese eel where phenylalanine requirement was 5.2% of protein when tyrosine was not included in the diet. However, the phenylalanine requirement was reduced by up to 2.9% with the inclusion of 2.0% tyrosine in the diet. Inclusion of adequate levels of tyrosine reduces the phenylalanine requirement. Hence, to satisfy the total aromatic amino acid requirement of fish, feeds should be formulated with required amount of phenylalanine and adequate tyrosine. Quadratic regression analysis of AWG, PRE, PHRE and RNA/DNA ratio against dietary phenylalanine concentrations yielded the requirement at 1.01% dry diet and against varying levels of dietary tyrosine reflected the requirement at 0.68% dry diet. Thus, it is recommended that inclusion of total aromatic amino acids at 1.69% (1.01% phenylalanine+0.68% tyrosine) dry diet, corresponding to 5.12% of dietary protein is optimum to maximize the growth of fingerling *C. catla.* Also, the tyrosine replacement value for phenylalanine in the present study was determined to be 40.2% on a weight basis or 36.7% on a molar basis. Data generated during the present study would be useful to formulate total aromatic amino acid balanced, cost-effective feeds for the culture of this fish species.

SUMMARY

Two 12 weeks experiments were conducted to determine the dietary total aromatic amino acid requirement and tyrosine replacement value for phenylalanine for fingerling *Catla catla*. In experiment I, phenylalanine requirement was determined by feeding six casein-gelatin based amino acid test diets (33% crude protein; 16.72 kJ/g gross energy) with graded levels of phenylalanine (0.39, 0.64, 0.87, 1.12, 1.38 and 1.62% dry diet) at a constant level (1%) of dietary tyrosine to triplicate groups of fish (3.95±0.24 cm; 0.68±0.19 g) near to satiation. Absolute weight gain (AWG g/fish), feed conversion ratio (FCR), protein retention efficiency (PRE%), phenylalanine retention efficiency (PHRE%) and RNA/DNA ratio responded positively with the increasing concentrations of phenylalanine reaching the highest values at 1.12% of the dry diet. Quadratic regression analysis of AWG, PRE, PHRE and RNA/DNA ratio at 95% of maximum response against varying levels of dietary phenylalanine exhibited the requirement at 1.02, 0.99, 0.97 and 1.06% dry diet, respectively. The above analysis revealed that inclusion of phenylalanine at 1.01% of dry diet, corresponding to 3.06% dietary protein is optimum. In Experiment II, six diets with different levels of L-tyrosine (0.19, 0.38, 0.59, 0.81, 0.98 and 1.18% dry diet) with 1.01% phenylalanine (determined in experiment I) fixed in all the test diets were fed to fish (3.85±0.25 cm; 0.66±0.16 g) to determine the tyrosine requirement under identical conditions. Quadratic regression analysis of AWG, PRE, PHRE and RNA/DNA ratio at 95% of maximum response against dietary tyrosine concentrations indicated the requirement at 0.64, 0.65, 0.71 and 0.73% dry diet, respectively. Hence, inclusion of tyrosine at 0.68% of the dry diet, corresponding to 2.06% of dietary protein is taken as the tyrosine required for optimum utilization of phenylalanine. Based on above data, total aromatic amino acid requirement of fingerling *C. catla* was found to be 1.69% (1.01% phenylalanine+0.68%tyrosine) of the dry diet and tyrosine replacement value for phenylalanine was found to be 36.7% on molar basis. It is recommended that fingerling *C. catla* require 1.69% TAAA of the dry diet (5.12% of dietary protein) of which 37% (on molar basis) could be supplied as tyrosine. Data generated during this study will be useful in formulating TAAA balanced, cost-effective commercial feeds for the intensive culture of this fish.

Table 1 Composition of the basal diets (%) used in experiment I and II

Ingredients	Basal diet (Experiment I)	Basal diet (Experiment II)
Casein[a] (fat-free)	7.15	3.1
Gelatin[b]	2.38	9
Dextrin	37.09	36.51
Amino acid mixture	23.12[c]	23.51[d]
Corn oil	5	5
Cod liver oil	2	2
Mineral mix[e,g]	4	4
Vitamin mix[f,g]	3	3
α- Cellulose	3.68	3.88
Carboxymethyl cellulose	10	10
Total	100	100
Analyzed crude protein (% dry diet)	32.83	32.91
Analyzed crude lipid (% dry diet)	7.1	7.0
Calculated gross energy (kJ/g, dry diet)	16.72	16.72

[a]Crude Protein (76%); [b]Crude Protein (96%); [c]Amino acid mixture (g/100 g) arginine 1.661, histidine 0.488, isoleucine 2.214, leucine 2.273, lysine 1.695, methionine 1.106, cystine 0.763, phenylalanine 0, tyrosine 0.631, threonine 1.133, tryptophan 0.437, valine 1.885, alanine 1.443, aspartic acid 0.554, glutamic acid 0.595, proline 1.703, serine 0.213, glycine 4.268 (Loba Chemie, India); [d]Amino acid mixture (g/100 g) arginine 1.267, histidine 0.540, isoleucine 2.372, leucine 2.498, lysine 1.766, methionine 1.211, cystine 0.779, phenylalanine 0.693, cystine 0; threonine 1.176 ; tryptophan 0.470, valine 2.058; alanine 0.8412, aspartic acid 0.4626, glutamic acid 0.8796, proline 1.173, serine 0.224; glycine 5.102 (Loba Chemie, India); [e,g]Mineral mixture (g/100 g of mineral premix) calcium biphosphate 13.57; calcium lactate 32.69; ferric citrate 2.97; magnesium sulphate 13.20; potassium phosphate (dibasic) 23.98; sodium biphosphate 08.72; sodium chloride 04.35; aluminium chloride. $6H_2O$ 0.0154; potassium iodide 0.015; cuprous chloride 0.010; manganous sulphate. H_2O 0.080; cobalt chloride. $6H_2O$ 0.100; zinc sulphate. $7H_2O$ 0.40; [f,g]Vitamin mixture (1 g vitamin mix+2 g α-cellulose) choline chloride 0.500; inositol 0.200; ascorbic acid 0.100; niacin 0.075; calcium pantothenate 0.05; riboflavin 0.02; menadione 0.004; pyridoxine hydrochloride 0.005; thiamine hydrochloride 0.005; folic acid 0.0015; biotin 0.0005; alpha-tocopherol 0.04; vitamin B_{12} 0.00001; [g]Halver (2002); Loba Chemie, India.

Table 2 Analyzed amino acid composition of the basal diets (%) to determine the phenylalanine and tyrosine requirement of fingerling *C. catla*[a]

	Basal diet (Experiment I)	Basal diet (Experiment II)
EAAs		
Arginine	2.11	2.10
Histidine	0.68	0.70
Isoleucine	2.63	2.65
Leucine	3.02	3.05
Lysine	2.36	2.34
Methionine	1.35	1.36
Phenylalanine	0.39	1.02
Threonine	1.42	1.42
Tryptophan	0.50	0.50
Valine	2.41	2.41
NEAAs		
Cystine	0.76	0.78
Tyrosine	0.98	0.19
Alanine	1.91	1.90
Aspartic acid	1.15	1.17
Glutamic acid	2.34	2.32
Glycine	5.01	7.44
Proline	2.75	2.73
Serine	0.58	0.59

[a]Determined by Hitachi L-8800 Automatic Amino Acid Analyzer

Table 3 Growth performance of fingerling *C. catla* fed diets containing varying levels of phenylalanine[a,b]

	Analyzed levels of phenylalanine (% dry diet)					
	0.39 (D1)	**0.64 (D2)**	**0.87 (D3)**	**1.12 (D4)**	**1.38 (D5)**	**1.62 (D6)**
Initial weight (g/fish)	0.68±0.01[a]	0.69±0.04[a]	0.68±0.02[a]	0.68±0.02[a]	0.67±0.05[a]	0.67±0.04[a]
Final weight (g/fish)	3.79±0.06[e]	5.79±0.09[d]	7.26±0.07[c]	8.91±0.11[a]	8.52±0.02[ab]	7.51±0.08[c]
Absolute weight gain (g/fish)	3.11±0.03[e]	5.10±0.06[d]	6.58±0.05[c]	8.23±0.08[a]	7.85±0.07[ab]	6.84±0.04[c]
Feed intake (g/fish)	11.54±0.17[b]	12.53±0.08[a]	12.77±0.09[a]	12.09±0.15[a]	12.40±0.14[a]	12.04±0.12[a]
Feed conversion ratio	3.71±0.02[a]	2.46±0.04[b]	1.94±0.06[c]	1.47±0.02[f]	1.58±0.04[e]	1.76±0.05[d]
Protein retention efficiency%	10.12±0.41[e]	18.51±0.23[d]	25.58±0.38[c]	35.99±0.29[a]	31.04±0.42[b]	25.62±0.27[c]
Phenylalanine retention efficiency%	36.94±1.93[f]	51.47±1.36[e]	64.39±1.64[cd]	76.53±1.53[a]	71.24±1.65[b]	62.83±1.74[c]
RNA/DNA ratio	1.67±0.04[f]	3.14±0.02[e]	4.21±0.03[d]	5.48±0.05[a]	5.21±0.04[b]	4.86±0.08[c]

[a]Mean values of 3 replicates ± SEM. [b]Mean values sharing the same superscript are not significantly different (P>0.05)

Table 4 Carcass compositions (wet basis) of fingerling *C. catla* fed diets containing varying levels of phenylalanine [a,b]

	Analyzed levels of phenylalanine (% dry diet)						
	Initial	**0.39 (D1)**	**0.64 (D2)**	**0.87 (D3)**	**1.12 (D4)**	**1.38 (D5)**	**1.62 (D6)**
Moisture (%)	79.34±0.94	79.21±0.91[a]	78.93±1.21[a]	77.89±0.95[b]	76.76±0.88[c]	75.11±0.93[d]	74.12± 0.99[e]
Protein (%)	11.67±0.24	12.26±0.25[d]	14.59±0.15[c]	15.94±0.13[b]	17.01±0.11[a]	15.84±0.19[b]	14.61±0.21[c]
Fat (%)	2.91± 0.21	2.45± 0.02[f]	2.89± 0.03[e]	3.26± 0.02[d]	3.68± 0.04[c]	4.67±0.06[b]	5.08± 0.05[a]
Ash (%)	2.24± 0.04	2.26±0.02[a]	2.24±0.02[a]	2.21± 0.01[a]	2.24±0.04[a]	2.25±0.02[a]	2.21± 0.03[a]
Survival (%)	-	94	100	100	100	100	100

[a]Mean value of 3 replicates ± SEM. [b]Mean values sharing the same superscript are not significantly different (P>0.05)

Table 5 Growth performance of fingerling *C. catla* fed diets containing varying levels of tyrosine[a,b]

	Analyzed levels of tyrosine (% dry diet)					
	0.19 (T1)	0.38 (T2)	0.59 (T3)	0.81 (T4)	0.98 (T5)	1.18 (T6)
Initial weight (g/fish)	0.66±0.02[a]	0.67±0.05[a]	0.66±0.02[a]	0.66±0.03[a]	0.66±0.07[a]	0.65±0.08[a]
Final weight (g/fish)	3.94±0.08[f]	5.96±0.12[e]	7.99±0.14[c]	9.68±0.07[a]	8.59±0.11[b]	6.96±0.07[d]
Absolute weight gain (g/fish)	3.28±0.06[e]	5.29±0.04[d]	7.33±0.08[b]	9.02±0.11[a]	7.93±0.09[b]	6.31±0.05[c]
Feed intake (g/fish)	11.94±0.13[b]	12.22±0.14[a]	12.75±0.11[a]	12.89±0.08[a]	12.76±0.12[a]	12.11±0.10[a]
Feed conversion ratio	3.64±0.04[a]	2.31±0.05[b]	1.74±0.06[d]	1.43±0.04[f]	1.61±0.05[e]	1.92±0.04[c]
Protein retention efficiency%	9.98±0.62[f]	18.43±0.59[e]	27.09±0.61[c]	36.76±0.53[a]	29.16±0.58[b]	22.63±0.62[d]
Phenylalanine retention efficiency%	34.62±1.08[e]	49.97±1.04[d]	63.25±1.13[c]	79.49±0.98[a]	75.71±1.25[b]	71.39±1.01[c]
RNA/DNA ratio	1.59±0.08[e]	2.92±0.03[d]	4.37±0.05[c]	5.21±0.04[a]	4.94±0.06[b]	4.72±0.04[c]

[a]Mean values of 3 replicates ± SEM. [b]Mean values sharing the same superscript are not significantly different (P>0.05)

Table 6 Carcass compositions (%wet basis) of fingerling *C. catla* fed diets containing varying levels of tyrosine[a,b]

	Analyzed levels of tyrosine (% dry diet)						
	Initial	**0.19 (T1)**	**0.38 (T2)**	**0.59 (T3)**	**0.81 (T4)**	**0.98 (T5)**	**1.18 (T6)**
Moisture (%)	78.68±0.92	78.94±2.8[a]	77.41±2.1[b]	76.68±2.4[c]	75.24±2.2[d]	75.94±2.4[d]	76.36± 2.1[d]
Protein (%)	11.94±0.21	11.99±0.19[d]	13.81±0.17[c]	15.25±0.18[b]	16.97±0.15[a]	15.21±0.14[b]	14.11±0.16[c]
Fat (%)	3.19± 0.16	2.98± 0.03[d]	3.46± 0.05[c]	3.91± 0.04[b]	4.75± 0.03[a]	4.67±0.06[a]	4.58± 0.05[a]
Ash (%)	2.15± 0.02	2.12±0.02[a]	2.16±0.04[a]	2.11± 0.03[a]	2.13±0.02[a]	2.12±0.04[a]	2.11± 0.02[a]
Survival (%)	-	100	100	100	100	100	100

[a]Mean value of 3 replicates ± SEM. [b]Mean values sharing the same superscript are not significantly different (P>0.05)

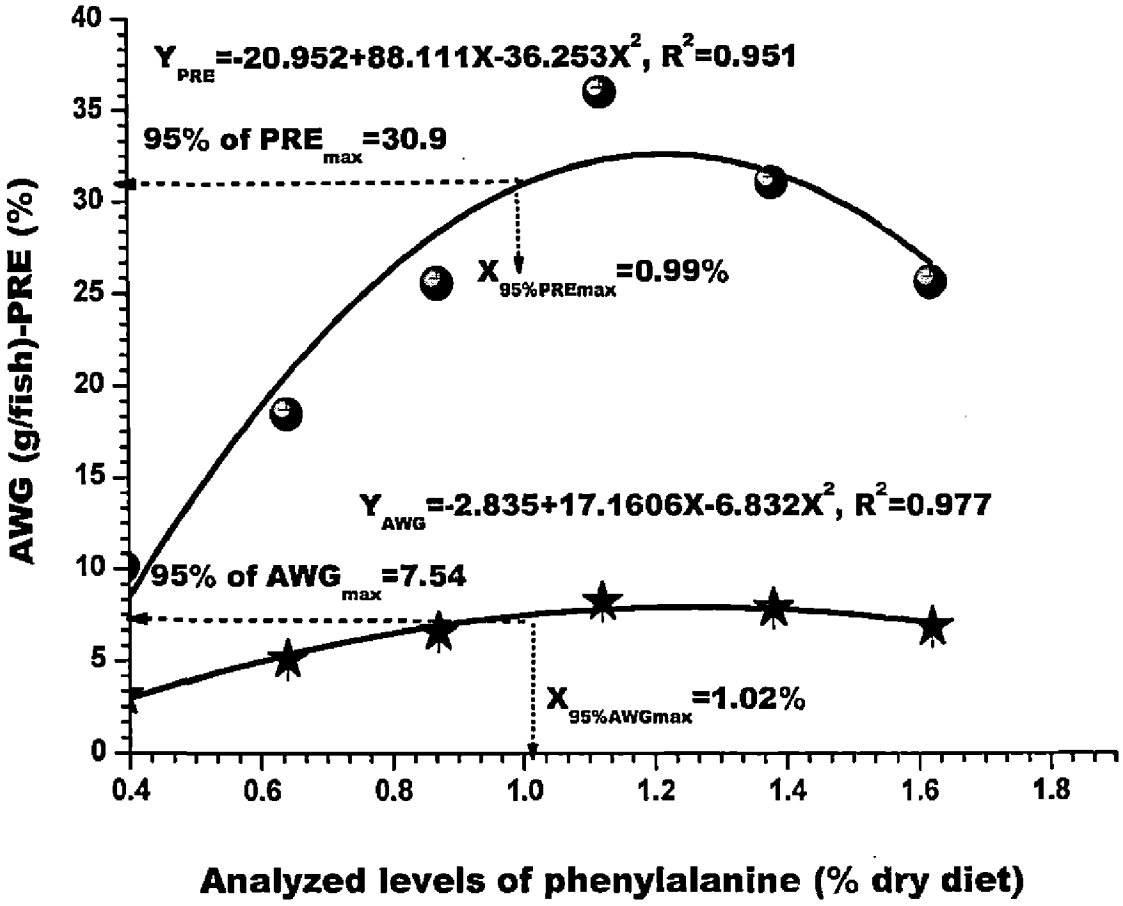

Fig. 1 Quadratic relationships of dietary phenylalanine to absolute weight gain and protein retention efficiency

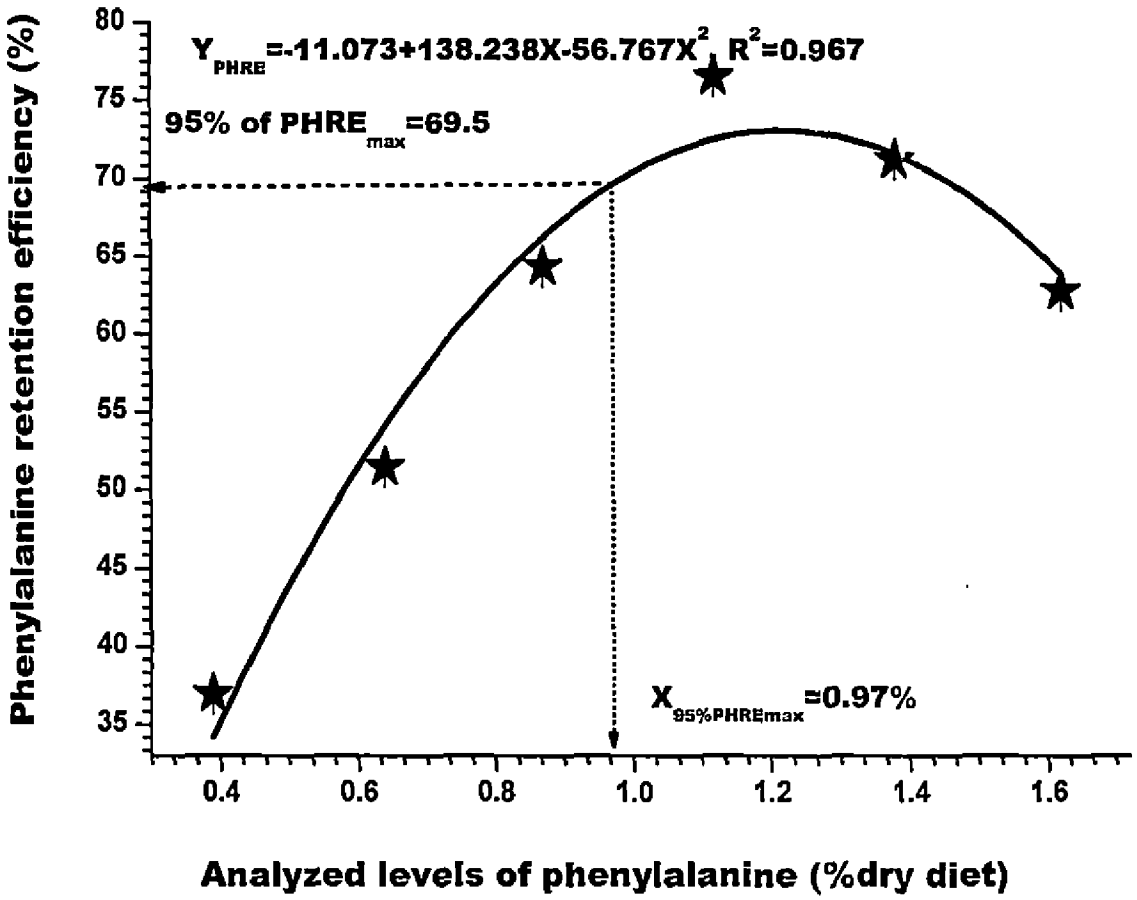

Fig. 2 Quadratic relationships of dietary phenylalanine to phenylalanine retention efficiency

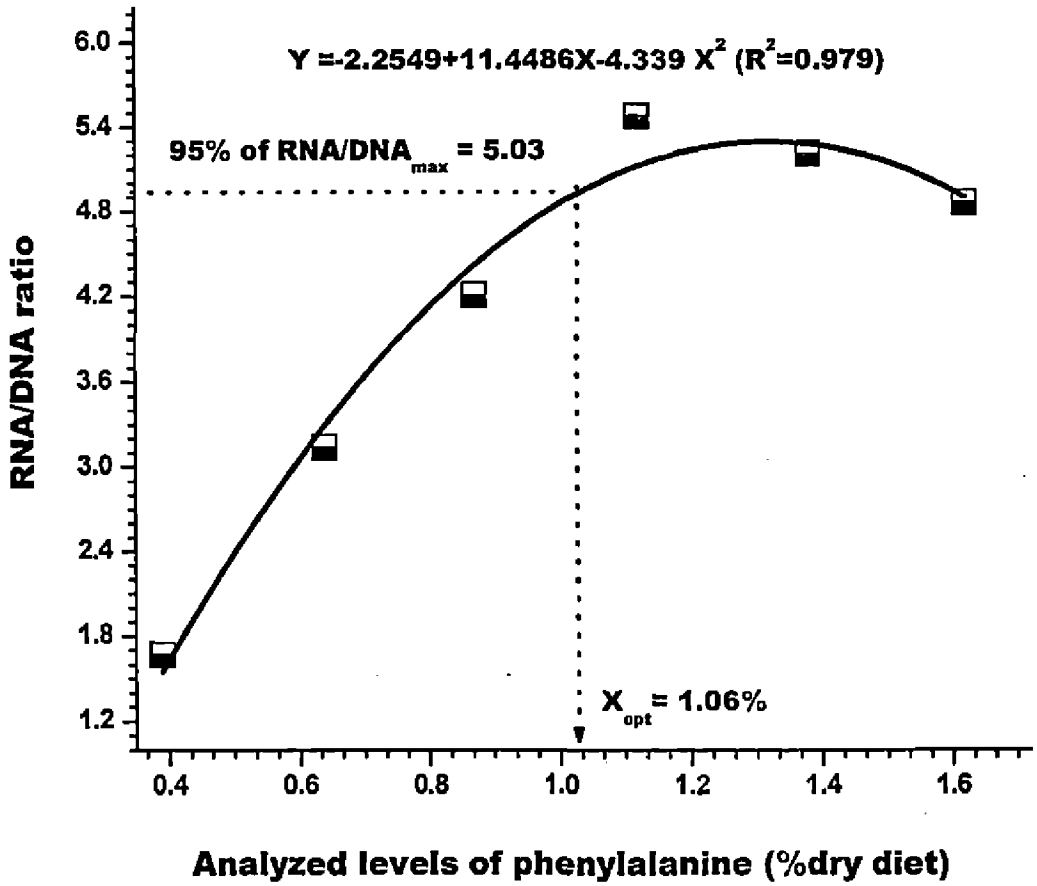

Fig. 3 Quadratic relationship of dietary phenylalanine to RNA/DNA ratio

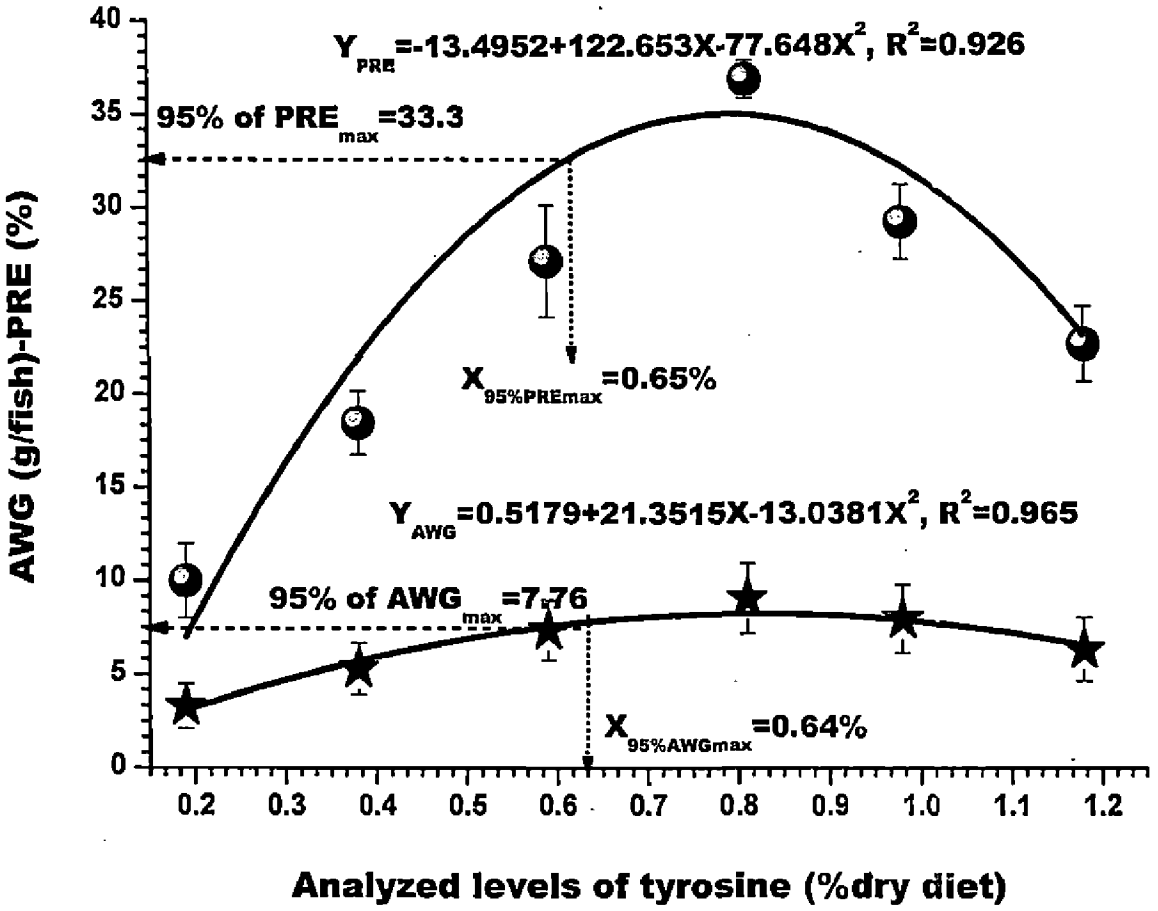

Fig. 4 Quadratic relationships of dietary tyrosine to absolute weight gain and protein retention efficiency

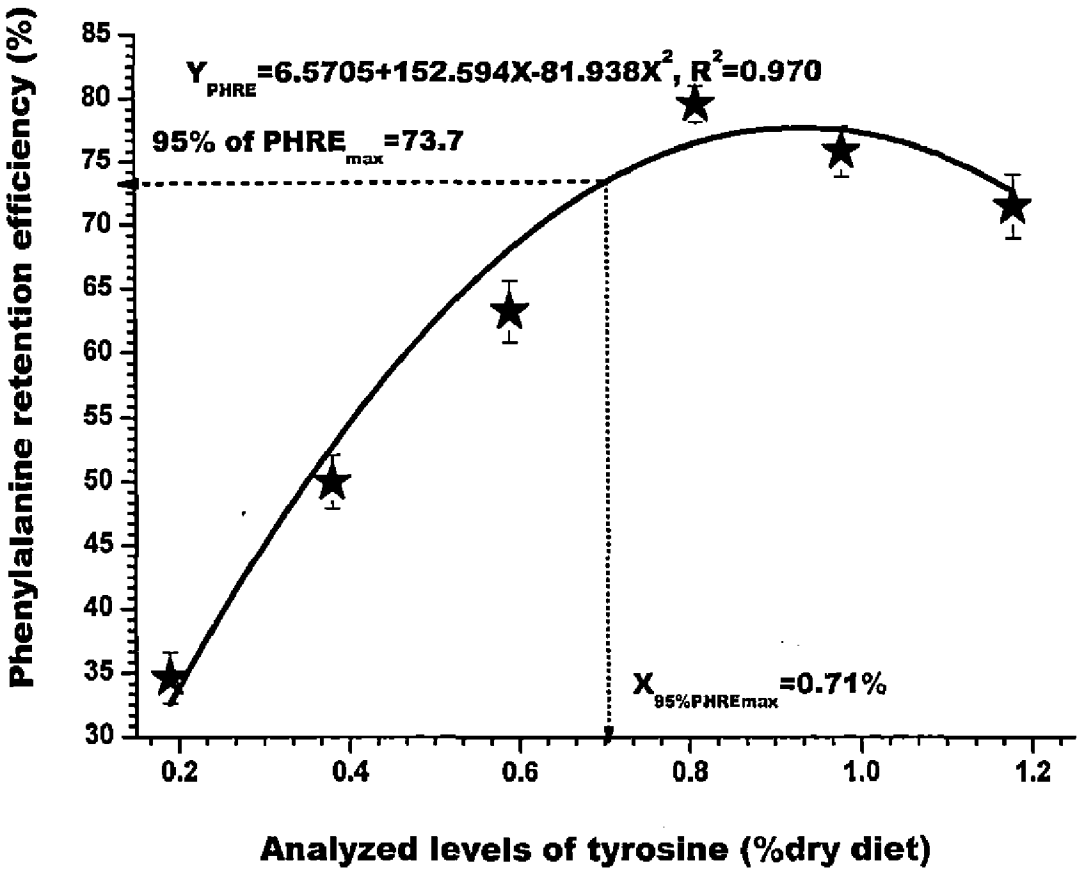

Fig. 5 Quadratic relationships of dietary tyrosine to phenylalanine retention efficiency

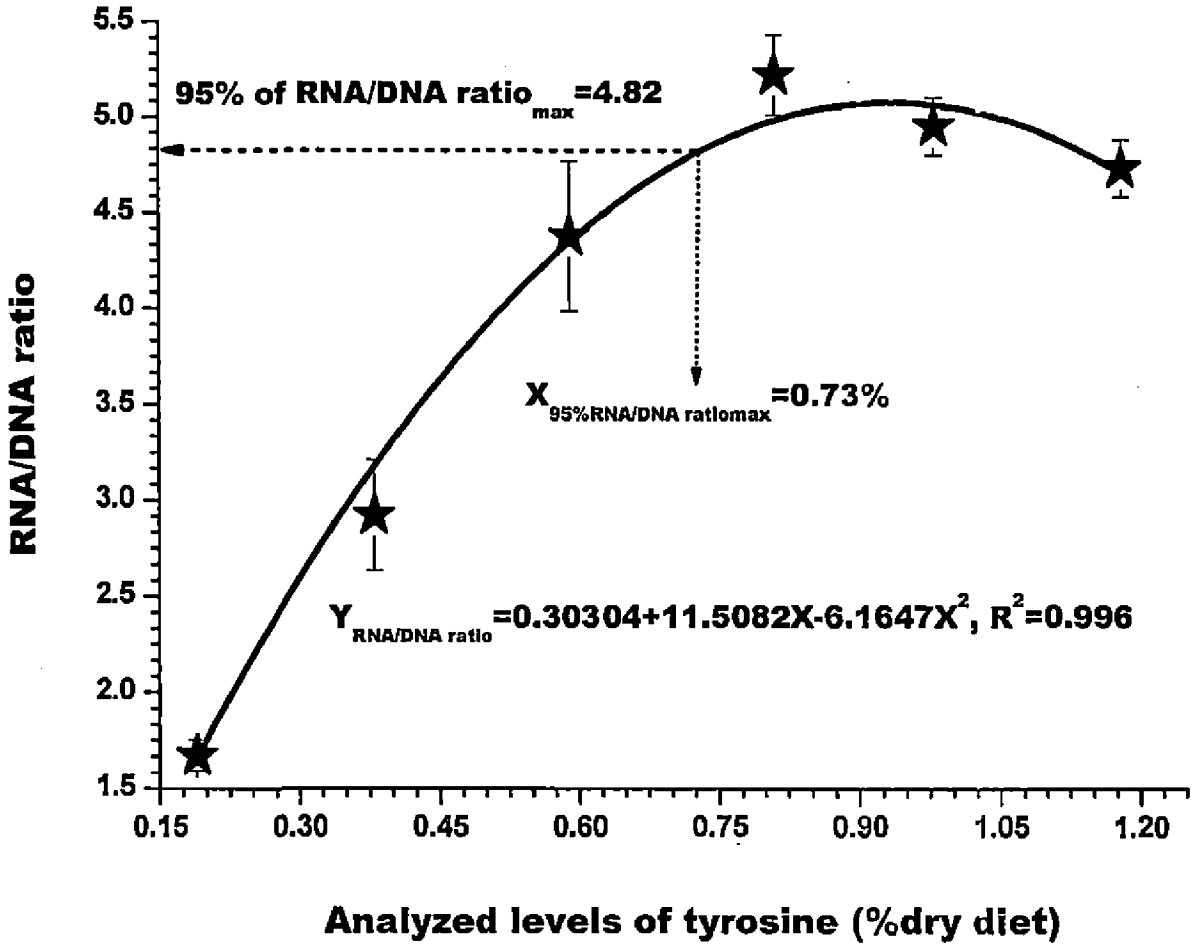

Fig. 6 Quadratic relationships of dietary tyrosine to RNA/DNA ratio

The data generated during the present study on essential amino acid requirements of fingerling *C. catla* have been summarized below:

Essential amino acids	**Requirements**	
	%dry diet	**%dietary protein**
Arginine	1.67	5.06
Lysine	1.7-1.8	5.2-5.5
Isoleucine	1.13-1.18	3.42-3.58
Valine	1.02	3.09
Leucine	1.57-1.59	4.76-4.82
Threonine	1.35-1.48	4.09-4.48
Tryptophan	0.21-0.25	0.64-0.76
Histidine	0.63-0.70	1.91-2.12
Total sulphur amino acids	1.28 (39% on equimolar sulphur basis could be spared by cystine)	3.87
Total aromatic amino acids	1.69 (37% on molar basis could be supplied as tyrosine)	5.12

The information generated during the present study on the quantitative essential amino acid requirements of *C. catla* would be useful in formulating amino acid balanced, cost-effective practical feeds for the intensive culture of this fish.

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JOURNAL OF THE
WORLD AQUACULTURE SOCIETY

Vol. 44, No. 3
June, 2013

Dietary Arginine Requirement of Fingerling Indian Major Carp, *Catla catla* (Hamilton)

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Abstract

Dietary arginine requirement of fingerling *Catla catla* (3.55 ± 0.05 cm; 0.61 ± 0.02 g) was determined by feeding casein–gelatin-based isonitrogenous (33% crude protein) and isocaloric (3.40 kcal/g digestible energy) amino acid test diets containing six graded levels of L-arginine (1, 1.25, 1.5, 1.75, 2, and 2.25% dry diet) for 12 wk. Maximum absolute weight gain (6.93 g/fish), protein efficiency ratio (2.13), protein deposition (0.36), arginine retention efficiency (78%), and best feed conversion ratio (1.42) were recorded in fish fed 1.75% arginine of the dry diet. Maximum carcass protein (15.57%) and RNA/DNA ratio (4.79) were also recorded for the group fed 1.75% arginine of the dry diet. Quadratic regression analysis at 95% maximum or minimum response of above growth parameters yielded optimum arginine requirement of fingerling *C. catla* at 1.67% of the dry diet. On the basis of the above analysis of the growth parameters, it is recommended that the inclusion of dietary arginine at 1.67% of the dry diet is optimum for formulating arginine-balanced, cost-effective quality feeds for the mass culture of fingerling *C. catla*.

In intensive culture systems, nutritionally complete feeds have been used and constitute a major proportion of aquaculture production cost. Hence, the development of cost-effective feeds is critical to the economic success of aquaculture system. Protein and amino acids are costliest components of fish feed (Nguyen and Davis 2009). Fish do not require protein *per se*, rather they require amino acids that comprise protein. Amino acids are not only important substrates for the synthesis of proteins and other nitrogenous compounds but also act as key regulators of fluxes through major metabolic pathways (Jobgen et al. 2006). Dietary deficiency of any essential amino acid will impair protein synthesis and suppress fish growth (Masagounder et al. 2010). Additionally, feeding excess concentrations of essential amino acid can result in increased ammonia excretion and degrade water quality (Hart et al. 2010). Therefore, to provide appropriate amounts of these nutrients in feeds, precise information on essential amino acids requirement of cultured species is crucial.

Arginine has important nutritional and physiological roles and is established as indispensable in the diet of many fish species (NRC 2011). In addition to as an important component of protein, arginine is involved in several biologically important metabolic pathways. It is a precursor of at least six biologically important compounds; thus, it is one of the most metabolically versatile amino acids (Morris 2006). Arginine affects metabolism of proteins, amino acids, glucose, and fatty acids and, therefore, the development (Flynn et al. 2002). It is an essential component of the urea cycle, which is the major pathway for elimination of ammonia in mammals (Flynn et al. 2002; Kohli 2003). In freshwater teleost, the activity of the urea cycle is very low compared with mammals (Depeche et al. 1979), and hence, the essentiality of arginine would be more pronounced in fish than in growing mammals. The presence of a full urea cycle in teleostean fish (Huggins et al. 1969) including rainbow trout (Chiu et al. 1988) suggests a potential for arginine biosynthesis. All species require substantial amounts of dietary arginine for maximum growth. This indicates that although an animal possesses the

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zyme necessary for arginine synthesis, the ..iount of arginine synthesized may not be nough to meet the need for maximum growth.

Dietary arginine requirements have been vorked out for many fish species such as .apanese flounder, *Paralichthys olivaceus* (Alam et al. 2002); mrigal, *Cirrhinus mrigala* (Ahmed and Khan 2004); hybrid Clarias, *Clarias gariepinus* × *C. macrocephalus* (Singh and Khan 2007); rohu, *Labeo rohita* (Abidi and Khan 2009); black sea bream, *Sparus macrocephalus* (Zhou et al. 2010); and stinging catfish, *Heteropneustes fossilis* (Khan and Abidi 2011).

Catla, *Catla catla*, the fish under study, is the fastest growing species among the three Indian major carps, the other two being rohu, *L. rohita*, and mrigal, *C. mrigala*. *C. catla*, a predominant zooplankton feeder, is an important component of polyculture with the above two species of carps. It has a great market demand because of its high nutritional value and good taste. Information on arginine requirement of fry *C. catla* is available (Ravi and Devaraj 1991). However, dietary arginine requirement of fingerling stage of this fish is completely lacking. As the arginine requirement reported by Ravi and Devaraj (1991) was based on weight gain only, which may also be owing to the deposition of fat or moisture content, it could not be an accurate parameter for nutrient requirement studies. Therefore, in addition to weight gain, this work reports the arginine requirement of fingerling based on sensitive parameters such as feed conversion, protein deposition (PD), arginine retention, RNA/DNA ratio, and carcass composition of fingerling *C. catla*.

Materials and Methods

Experimental Diet

Six isonitrogenous (33% crude protein) and isocaloric (3.40 kcal/g digestible energy) amino acid test diets with graded levels of arginine (1, 1.25, 1.5, 1.75, 2, and 2.25% dry diet) were prepared using casein (vitamin and fat-free), gelatin, and L-crystalline amino acids (Table 1). The levels of arginine in the amino acid test diets were fixed on the basis of information available on other carps (Ahmed and Khan 2004; Abidi and Khan 2009; NRC 2011). The amino acid compositions of the experimental diets, excluding the test amino acid arginine, were simulated to that of 33% whole chicken egg protein. To ensure maximum utilization of the limiting amino acids (Wilson 2002), the dietary protein level in this study was fixed at above level, which is slightly lower than 35% reported earlier (Khan and Jafri 1991; Dars et al. 2010). The arginine content contributed by the casein and gelatin was 0.54 and 0.41% of the dry diet, respectively. To make the intended concentrations of dietary arginine in the amino acid test diets, the amount of arginine was increased at the expense of glycine on protein basis. Diets were made isonitrogenous and isoenergetic by adjusting glycine and the dextrin. The amino acids mixture was coated with casein, gelatin, and carboxymethyl cellulose to minimize the leaching and maximize the gut retentivity of amino acids. Method of preparation of experimental diets was the same as detailed earlier (Abidi and Khan 2007). Briefly, the preweighed quantities of L-crystalline amino acids and salt mixture were thoroughly stirred in hot water (80 C) in a steel bowl attached to a Hobart electric mixer (K5SS, Hobart Corp., Troy, OH, USA). The pH of the resulting mixture was adjusted to neutral with 6N NaOH solution (Nose et al. 1974). Gelatin was dissolved separately in a volume of water with constant heating and stirring and then transferred to the above mixture for coating of the L-crystalline amino acids. This gelatin-coated amino acids mixture was further coated with cooked casein at 80 C. The mixer bowl was removed from heating and dextrin added. Vitamin and oil premixes, excepting carboxy methylcellulose, were added to the lukewarm bowl (50 C) one by one with constant mixing. Carboxy methylcellulose was added in the last, and the speed of the blender was gradually increased as the diet began to harden. The dough was passed through a pelletizer fitted with a 2-mm die to obtain pellets, which were dried in a hot air oven at 40 C to reduce the moisture content below 10%. The dry pellets thus obtained

TABLE 1. *Composition of experimental diets for fingerling* Catla catla *(initial body weight = 0.61 ± 0.02 g/fish) for 12 wk.*

	Dietary arginine levels (% dry diet)					
Ingredients (g/100 g dry diet)	1.00	1.25	1.50	1.75	2.00	2.25
Casein[a] (fat-free)	15	15	15	15	15	15
Gelatin[b]	5	5	5	5	5	5
Dextrin	32.27	32.55	32.82	33.10	33.37	33.65
Amino acid mixture[c]	18.32	18.14	17.96	17.78	17.60	17.42
Corn oil	5	5	5	5	5	5
Cod liver oil	2	2	2	2	2	2
Mineral mix[d,e]	4	4	4	4	4	4
Vitamin mix[e,f]	3	3	3	3	3	3
α-Cellulose	5.41	5.31	5.22	5.13	5.03	4.94
Carboxymethyl cellulose	10	10	10	10	10	10
Total	100	100	100	100	100	100
Calculated gross energy[g] (kcal/g, dry diet)	4.0	4.0	4.0	4.0	4.0	4.0
Estimated gross energy (kcal/g, dry diet)	3.87	3.81	3.92	3.85	3.89	3.97
Digestible energy[h] (kcal/g, dry diet)	3.38	3.39	3.39	3.41	3.42	3.43

[a]Crude protein (76%); Loba Chemie, Mumbai, India; arginine 0.54% of dry diet.

[b]Crude protein (96%); Loba Chemie; arginine 0.41% of dry diet.

[c]Amino acid mixture (g/100 g dry diet): arginine variable; histidine 0.26; isoleucine 1.747; leucine 1.44; lysine 0.953; methionine 0.789; cystine 0.73; phenylalanine 1.24; tyrosine 0.67; threonine 0.768; tryptophan 0.38; valine 1.313; alanine 0.94; proline 0.527; and glycine variable (Loba Chemie).

[d]Mineral mixture (g/100 g of mineral mix): calcium biphosphate 13.57; calcium lactate 32.69; ferric citrate 02.97; magnesium sulfate 13.20; potassium phosphate (dibasic) 23.98; sodium biphosphate 08.72; sodium chloride 04.35; aluminum chloride·$6H_2O$ 0.0154; potassium iodide 0.015; cuprous chloride 0.010; magnus sulfate·H_2O 0.080; cobalt chloride·$6H_2O$ 0.100; and zinc sulfate·$7H_2O$ 0.40.

[e]Halver (2002); Loba Chemie.

[f]Vitamin mixture (mg/100 g of dry diet): choline chloride 500; inositol 200; ascorbic acid 100; niacin 75; calcium pantothenate 50; riboflavin 20; menadione 4; pyridoxine hydrochloride 5; folic acid 1.5; biotin 0.5; α-tocopherol 40; vitamin B_{12} 0.01; and 2000 mg α-cellulose.

[g]Calculated on the basis of fuel values 5.52, 4.83, 3.83, 5.81, and 9.0 kcal/g for casein, gelatin, dextrin, amino acid, and fat, respectively, as estimated on Gallenkamp ballistic bomb calorimeter-CBB 330 010L (Gallenkamp).

[h]Digestible energy was calculated on the basis of physiological fuel values 4.5, 3.5, and 8.51 kcal/g for protein, carbohydrate, and fat, respectively (Jauncey 1982).

were crumbled, sieved (0.20–0.25 mm), and stored at 4 C until used. Water stability of the diet was estimated as per the method adopted by Fagbenro and Jauncey (1995), which was checked and found to be 97%.

Experimental Design and Feeding Trial

Induced bred fry *C. catla* were procured from G. B. Pant University of Agriculture and Technology, Pantnagar. These were then transported to the wet laboratory, given a prophylactic dip in $KMnO_4$ solution (1:3000) before stocking in indoor circular aqua-blue colored, plastic-lined (Plastic Crafts Corp, Mumbai, India) fish tanks (1.22 × 0.91 × 0.91 m; water volume 600 L) for 2 wk. During this period, the fish were acclimated to a casein–gelatin-based (33% CP) H-440 diet (Halver 2002) and reared to fingerling stage.

Fingerling *C. catla* (3.55 ± 0.05 cm; 0.61 ± 0.02 g) were taken from the above acclimated fish lot and stocked in triplicate groups at the rate of 25 fish per trough in 70-L circular polyvinyl troughs (water volume 55 L) fitted with a continuous water flow-through (1–1.5 L/min) system. Fish were fed test diets to apparent satiation in the form of dry crumbles (0.20–0.25 mm) thrice daily at 0800, 1230, and 1730 h. Initial and weekly weights were recorded on a top-loading balance (Precisa 120A; 0.1 mg sensitivity, Oerlikon AG, Zurich, Switzerland) after anesthetizing the

fish with tricaine methane sulfonate (MS-222; 100 µg/mL). Fish were deprived of feed on the day the measurements were taken. The feeding trial lasted for 12 wk. Fecal matter was siphoned before every feeding. Water quality parameters were monitored daily during the feeding trial and recorded following standard methods (APHA 1992). The average water temperature, dissolved oxygen, free carbon dioxide, pH, total ammonia nitrogen, nitrites, and total alkalinity, based on daily measurements, were 27.2–28.6 C, 6.8–7.3 mg/L, 5.5–9.3 mg/L, 7.3–7.6, 0.31–0.35 mg/L, 0.04–0.08 mg/L, and 65.4–82.6 mg/L, respectively.

Chemical Analyses

Proximate composition of experimental diets, and initial and final whole-body carcass were estimated using standard methods (AOAC 1995) for dry matter (oven drying at 105 ± 1 C for 22 h), crude protein (Kjeltec TecatorTM Technology 2300, Hoganas, Sweden), crude fat (solvent extraction with petroleum ether B.P 40–60 C for 2–4 h using Socs Plus, SCS 4, Pelican equipments, Chennai, India), and ash (oven incineration at 650 C for 2–4 h using muffle furnace, S.M. Scientific Instrument (p) Ltd. Jindal Company, New Delhi, India). Gross energy content was determined on a Gallenkamp Ballistic Bomb Calorimeter-CBB 330 010L (Gallenkamp, Loughbrough, UK). Amino acid analysis of casein, gelatin, and experimental diets (Table 2), and initial and final fish carcass were performed by hydrolyzing 0.3 mg sample in 1 mL of 6N HCl for about 22 h. The sample thus obtained was diluted in 0.02N HCl and injected into an automatic Amino Acid Analyzer (Hitachi L-8800, Tokyo, Japan). Recovery hydrolysis was performed in 4N methanesulfonic acid for the analysis of tryptophan and in performic acid for the recovery of sulfur amino acids. At the beginning of the feeding trial, 60 fish were randomly sampled, anesthetized in MS-222 (100 µg/mL), killed, and pooled together. Six subsamples of a pooled sample were analyzed for initial whole-body carcass composition. At the end of the experiment, 20 fish from each replicate of dietary treatments were randomly killed by dipping in chilled water and pooled separately. Three subsamples of the pooled samples were analyzed for final biochemical composition.

TABLE 2. *Analyzed amino acid profile of the experimental diets (% dry diet) for fingerling* Catla catla *(initial body weight = 0.61 ± 0.02 g/fish) for 12 wk.*[a]

Amino acid	1.00	1.25	1.50	1.75	2.00	2.25
Essential amino acids						
Arginine	0.98	1.23	1.47	1.72	1.97	2.23
Histidine	0.70	0.71	0.69	0.70	0.72	0.69
Isoleucine	2.66	2.65	2.65	2.66	2.68	2.67
Leucine	3.11	3.13	3.10	3.12	3.11	3.12
Lysine	2.42	2.40	2.39	2.41	2.40	2.38
Methionine	1.37	1.35	1.38	1.37	1.36	1.37
Phenylalanine	2.12	2.13	2.11	2.14	2.10	2.11
Threonine	1.47	1.45	1.45	1.44	1.46	1.47
Tryptophan	0.53	0.52	0.53	0.53	0.51	0.52
Valine	2.43	2.44	2.46	2.41	2.42	2.44
Nonessential amino acids						
Cystine	0.81	0.83	0.80	0.82	0.81	0.83
Tyrosine	1.51	1.52	1.53	1.50	1.51	1.52
Alanine	1.89	1.91	1.88	1.92	1.87	1.88
Aspartic acid	1.16	1.15	1.14	1.13	1.16	1.17
Glycine	7.91	7.51	7.09	6.65	6.21	5.78
Proline	2.81	2.83	2.80	2.82	2.83	2.84
Serine	0.55	0.56	0.54	0.52	0.55	0.54

[a]Determined by Automatic Amino Acid Analyzer (Hitachi L-8800).

Determination of RNA and DNA

The RNA and DNA were determined by the method of Schneider (1957). After the termination of feeding trial, three fish from each replicate of the treatment group ($n = 3 \times 3$) were killed by dipping in chilled water and white muscle tissue was taken for analysis. Three subsamples of the tissue samples from each replicate of the treatment group were taken for the determination of RNA/DNA ratio. The dry matter of the white muscle was analyzed, which was found to be 21.37%. Muscle samples (100 mg) were homogenized in 5% trichloroacetic acid for 5 min at 90 C and then centrifuged at 3354 *g* for 20 min. For the determination of RNA, 2.0 mL of distilled water and 3.0 mL of orcinol reagent were added in 1.0 mL of supernatant. The reaction mixture was kept in boiling water bath for 20 min. The greenish-blue color thus developed was read at 660 nm in a spectrophotometer (Genesis 10-UV, Thermo Spectronic, Madison, WI, USA). For DNA determination, 1.0 mL of distilled water and 4.0 mL of freshly prepared diphenylamine reagent were added to 1.0 mL of the supernatant. The reaction mixture was kept on a boiling water bath for 10 min. The blue color developed was measured at 600 nm. Standard curves for RNA and DNA were drawn using different concentrations of yeast RNA and calf thymus DNA, respectively. The values were expressed as µg/100 mg fish muscle tissue on dry basis.

Data Analyses

Growth performance of the experimental diets was measured as a function of the weight gain by calculating the following parameters:

Absolute weight gain (g/fish)

= Final body weight-Initial body weight

Protein efficiency ratio

= Weight gain (g) /Protein intake (g)

Feed intake (g/fish) = Total dry feed intake (g)

/Total no. of fish

Feed conversion ratio

=Dry feed intake (g) /Wet weight gain (g)

Protein deposition

= Protein gain (g) /Protein intake (g)

Arginine retention efficiency%

= Arginine gain/Arginine intake × 100

Statistical Analyses

Dietary arginine requirement of fingerling *C. catla* was estimated by the quadratic regression analysis of the growth parameters at 95% of maximum or minimum response (Shearer 2000). Determination of the significance of the trend of these responses was assessed using quadratic contrasts of dietary arginine at a significance level of $P < 0.05$. All the statistical analyses were performed using Origin (version 6.1; Origin Software, San Clemente, CA, USA).

Results

Data for absolute weight gain (AWG; g/fish), protein efficiency ratio (PER), feed conversion ratio (FCR), PD, arginine retention efficiency (ARE; %), and feed intake are given in Table 3. These parameters were affected by varying concentrations of dietary arginine. Fish receiving 1.75% dietary arginine reflected best AWG (6.93 g/fish), PER (2.20), and FCR (1.42). Similarly, highest PD (0.36) and ARE (78%) were recorded at this level. Feed intake remained almost constant among the treatments. Fish fed diets containing more than 1.75% dietary arginine exhibited reduced growth in terms of weight gain, feed conversion, PD, and arginine retention. Fish fed diet with 1% arginine showed poorest AWG (2.77 g/fish), PER (0.84), FCR (3.71), PD (0.11), and ARE (38%). Survival in all the treatments was found to be 100% irrespective of the dietary arginine levels.

To generate more precise data on arginine requirement of fingerling *C. catla*, all the growth data were subjected to quadratic regression analysis at 95% of maximum or minimum response. Quadratic regression analysis of AWG (Fig. 1), FCR, PER, PD, and ARE against

TABLE 3. *Growth, conversion efficiency, protein deposition, and arginine retention of fingerling* Catla catla *(initial body weight = 0.61 ± 0.02 g/fish) fed diets containing varying levels of dietary arginine for 12 wk.*[a]

	Dietary arginine levels (% dry diet)						
	1.00	1.25	1.50	1.75	2.00	2.25	Quadratic Pr > F[c]
Average initial weight (g)	0.58 ± 0.02	0.61 ± 0.03	0.60 ± 0.05	0.59 ± 0.02	0.61 ± 0.08	0.61 ± 0.08	0.507[b]
Average final weight (g)	3.35 ± 0.07	5.27 ± 0.07	6.49 ± 0.14	7.32 ± 0.09	7.27 ± 0.13	6.49 ± 0.13	0.0001
Absolute weight gain (g/fish)	2.77 ± 0.04	4.66 ± 0.06	5.89 ± 0.05	6.73 ± 0.07	6.66 ± 0.02	5.88 ± 0.10	0.0001
Feed intake (g/fish)	10.28 ± 0.08	10.95 ± 0.11	10.07 ± 0.13	9.56 ± 0.14	10.72 ± 0.13	10.99 ± 0.09	0.540[b]
Feed conversion ratio (g/g)	3.71 ± 0.03	2.35 ± 0.04	1.71 ± 0.02	1.42 ± 0.02	1.61 ± 0.02	1.87 ± 0.02	0.001
Protein efficiency ratio (g/g)	0.82 ± 0.01	1.29 ± 0.03	1.77 ± 0.13	2.13 ± 0.12	1.88 ± 0.02	1.62 ± 0.06	0.007
Protein deposition (g/g)	0.11 ± 0.01	0.19 ± 0.01	0.27 ± 0.02	0.34 ± 0.02	0.28 ± 0.01	0.23 ± 0.01	0.014
Arginine retention efficiency%	38 ± 1.3	49 ± 1.1	57 ± 2.4	78 ± 1.6	71 ± 1.9	62 ± 1.2	0.049

[a]Mean values of three replicates ± SEM.
[b]Not statistically significant ($P > 0.05$).
[c]Significance probability associated with the F statistic.

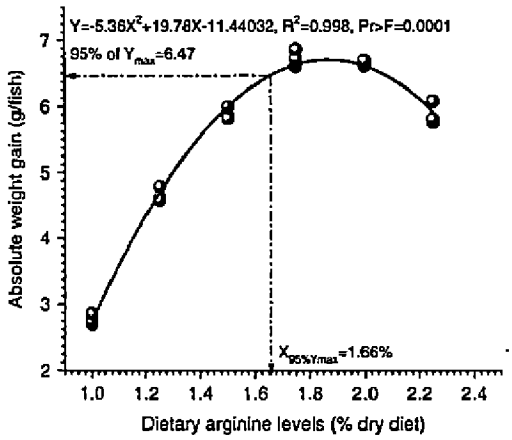

FIGURE 1. *Quadratic relationship of absolute weight gain to dietary arginine concentrations in fingerling* Catla catla *(initial body weight = 0.61 ± 0.02 g/fish) for 12 wk.*

dietary arginine concentrations exhibited the optimum arginine requirement of fingerling *C. catla* between 1.64 and 1.69% of the dry diet.

Carcass composition of fingerling *C. catla* was also affected quadratically in response to varying levels of arginine in the diets excepting carcass ash content (Table 4). Carcass protein content in fish fed diet containing 1.75% arginine was higher compared with the groups receiving other dietary arginine concentrations at 1, 1.25, 1.5, 2, and 2.25%. The quadratic relationship of carcass protein to dietary arginine is given in Table 5. According to this model, at 95% of the asymptote of carcass protein the calculated optimal arginine requirement was found to be 1.65% of the dry diet. Carcass fat decreased linearly (4.68–2.84%) with the increase in dietary arginine levels from 1 to 2.25%. Moisture content was found to be negatively correlated with the carcass fat. Ash content remained almost unchanged among varying test groups. The DNA content of the white muscle sample was found to decrease from 1 to 1.75% of the dietary arginine. However, the muscle RNA and RNA/DNA ratio improved with the increased inclusion of dietary arginine up to 1.75% (Table 4). The data for RNA/DNA ratio were also subjected to quadratic regression analysis, which at 95% of maximum response exhibited the arginine requirement at 1.66% of the dry diet.

On the basis of the above results, the arginine requirement of fingerling *C. catla* is recommended to be 1.67% of the dry diet, which is taken as a mean of arginine requirement reflected by several response variables. The equations employed for each response variables have been summarized in Table 5.

Discussion

During this study, maximum growth was noted in fish fed diet containing 1.75% arginine. However, fish fed diets containing more than the above level of dietary arginine (2–2.25%) reflected reduction in growth. Similar growth-depressing effect in fish fed higher doses of arginine was also recorded in other fish species such as mrigal (Ahmed and Khan 2004), hybrid clarias (Singh and Khan 2007), and rohu (Abidi and Khan 2009). This growth depression may

TABLE 4. *Carcass composition (% wet basis) and RNA/DNA ratio of fingerling* Catla catla *(initial body weight = 0.61 ± 0.02 g/fish) fed diets containing varying levels of dietary arginine for 12 wk.*[a]

	Dietary arginine levels (% dry diet)							
	Initial	1.00	1.25	1.50	1.75	2.00	2.25	Quadratic Pr > F[d]
Moisture (%)	79.12 ± 0.58	75.27 ± 0.52	76.14 ± 0.41	76.62 ± 0.46	77.24 ± 0.59	77.69 ± 0.51	78.15 ± 0.42	0.0001
Protein (%)	12.47 ± 0.16	12.49 ± 0.06	13.54 ± 0.13	14.68 ± 0.07	15.57 ± 0.11	14.63 ± 0.12	13.78 ± 0.08	0.017
Fat (%)	2.82 ± 0.11	4.68 ± 0.08	4.11 ± 0.04	3.55 ± 0.03	3.01 ± 0.03	2.98 ± 0.02	2.84 ± 0.05	0.001
Ash (%)	2.16 ± 0.08	2.13 ± 0.02	2.11 ± 0.01	2.12 ± 0.02	2.10 ± 0.02	2.11 ± 0.03	2.12 ± 0.02	0.239[b]
RNA[c]	622 ± 4.35	597 ± 4.12	838 ± 5.24	921 ± 6.11	1184 ± 6.12	1102 ± 5.86	996 ± 4.71	0.02
DNA[c]	426 ± 2.41	433 ± 2.54	312 ± 2.92	277 ± 3.83	247 ± 1.63	264 ± 2.73	269 ± 2.54	0.008
RNA/DNA ratio	1.46 ± 0.04	1.38 ± 0.02	2.69 ± 0.03	3.32 ± 0.05	4.79 ± 0.07	4.18 ± 0.04	3.71 ± 0.02	0.018

[a]Mean values of three replicates ± SEM.
[b]Not statistically significant ($P > 0.05$).
[c]µg RNA or DNA/100 g, dry weight basis.
[d]Significance probability associated with the F statistic.

TABLE 5. *Quadratic equations for growth parameters of fingerling* Catla catla *(initial body weight = 0.61 ± 0.02 g/fish) fed diets containing varying levels of dietary arginine for 12 wk.*[a]

Growth parameters	*a*	*b*	*c*	Arginine requirement[b]	R^2
AWG	−5.36	19.78	−11.44032	$X_{opt} = 1.66\%$	0.998
FCR	3.26286	−11.9425	12.3075	$X_{opt} = 1.69\%$	0.986
PER	−1.80571	6.59771	−4.03057	$X_{opt} = 1.64\%$	0.963
PD	−0.30264	1.10237	−0.69897	$X_{opt} = 1.66\%$	0.969
ARE%	−48.7143	172.257	−91.6628	$X_{opt} = 1.64\%$	0.866
RNA/DNA ratio	−3.96	14.8803	−9.657	$X_{opt} = 1.66\%$	0.930
Carcass protein	−3.76	14.29857	−1.85357	$X_{opt} = 1.65\%$	0.967

ARE, arginine retention efficiency; AWG, absolute weight gain; FCR, feed conversion ratio; PD, protein deposition; PER, protein efficiency ratio.
[a]Coefficient of the quadratic equations ($Y = aX^2 + bX + c$) and optimum level of arginine at 95% of maximum or minimum response.
[b]Requirement as % of dry diet.

be owing to the stress caused by the excess amount of amino acids in the body of the fish leading to extra energy expenditure toward deamination and excretion of the same (Walton 1985). The reduction in growth at higher levels of dietary arginine could also be attributed to amino acid toxicity. The accumulation of an amino acid or its degradative products in body pools may stress enzymatic systems and lead to further accumulation and possible toxicity (Alam et al. 2003), which may have an adverse effect on growth because disproportionate intake affects absorption and utilization of other amino acids or decreases the diet's palatability (Borlongan and Coloso 1993). Also, it has been reported that the major proportion of dietary limiting amino acids is used for protein synthesis, while amino acids in excess will be more available for oxidation (Gahl et al. 1996), which may be the reason for growth depression at higher levels of dietary arginine.

Dietary arginine supplementation can beneficially increase protein gain and reduce carcass fat accretion (Tan et al. 2009). Carcass protein content was found to be maximum in fish fed diet containing 1.75% arginine. Further inclusion of dietary arginine showed reduction in carcass protein content, indicating that the amount of arginine exceeding this level might have not been used for increasing body protein synthesis. Arginine supplementation increases lipolysis and inhibits lipogenesis by modulating the expression and function of key enzymes that are involved in antioxidative response and fat metabolism in insulin-sensitive tissues (Jobgen et al. 2009). In this study, carcass fat content responded negatively with the increase in dietary arginine concentrations. The decrease in carcass fat with the increasing concentrations of dietary arginine, as evident in this study,

was also reported by Khan and Abidi (2011) in stinging catfish. Arginine also plays a crucial role in regulating extra-endocrine signaling pathways such as AMP-activated protein kinase pathway (Yao et al. 2008), which has inhibitory effects on biosynthetic pathways for fatty acid and sterol synthesis (Viollet et al. 2003; Zhou et al. 2010) leading to linear reduction in carcass fat in this study.

The RNA/DNA ratio is considered to be a useful and reliable indicator of fish growth. The quantity of DNA in an animal cell is believed to be normally stable, but the quantity of RNA is closely related to the rate of body protein synthesis (Tanaka et al. 2007). As protein production varies in accordance with the quantity of RNA, the RNA/DNA ratio has been used to evaluate growth of many fishes (Mustafa 1977; Mustafa and Mittal 1982; Mustafa and Zofair 1985; Bulow 1987; Mustafa et al. 1991; Peck et al. 2003; Mercaldo-Allen et al. 2008). The RNA/DNA ratio in fingerling *C. catla* exhibited a quadratic response pattern to increasing concentrations of dietary arginine and improved up to 1.75% arginine of the dry diet. Further increment in dietary arginine (2–2.25%) showed a decline in RNA/DNA ratio. Almost similar response was also reported by Abidi and Khan (2009) in *L. rohita*.

As PD and amino acid retention reflect true picture of an amino acid requirement, these parameters were also used in this study to work out the arginine requirement of this fish. The quadratic regression analysis at 95% of maximum response of growth parameters indicated the optimum arginine requirement of fingerling *C. catla* to be at 1.67% of the dry diet. This requirement is higher than that reported for channel catfish 1.2% (NRC 2011); rainbow trout 1.5% (NRC 2011); and rohu 1.22–1.39% (Abidi and Khan 2009) and lower than that reported for catla 1.92% (Ravi and Devaraj 1991); Japanese flounder 2.25% (Alam et al. 2002); mrigal 1.8% (Ahmed and Khan 2004); coho salmon, *Oncorhynchus kisutch* 2.2% (NRC 2011); and black sea bream 2.8–3.1% (Zhou et al. 2010), and approximately comparable to common carp, *Cyprinus carpio* 1.7% (Nose 1979); Atlantic salmon 1.6% (Lall et al. 1994); and yellow perch 1.6% of the dry diet (Twibell and Brown 1997). It has been suggested that the wide variability and the reliability of arginine requirements of fish may be affected by fish size and age, feeding regime, feed allowance, adequate levels of other nutrients, water temperature, flow rate, stock density, and environmental and culture conditions adopted in different laboratories (Chiu et al. 1988; Luzzana et al. 1998; Hansen et al. 2010). Digestibility, amino acid profile, and energy content may also bring about variable effects in amino acid requirement studies (Simmons et al. 1999; De Silva et al. 2000). Response criteria and the mathematical model used to estimate the optimal value may also affect the arginine requirements of fish (Zhou et al. 2010).

The arginine requirement of fingerling *C. catla* worked out during this study (1.67% of the dry diet) is lower than that reported by Ravi and Devaraj (1991) for fry stage of this fish (1.92% of the dry diet). Higher amino acid requirement estimates are associated with the smaller size fish, whereas it becomes comparatively lower with the advancing stages because smaller size fish have higher rate of metabolism compared with larger size (Conklin 2000). This may probably be the reason for the differences in the arginine requirement as fish under study was in fingerling stage requiring lower dietary nutrient requirements for the metabolical and physiological activities than the fry stage of the fish in the study conducted by Ravi and Devaraj (1991). Also, they adopted restricted feeding strategy in their experiments, whereas in this study, satiation feeding at three feeding frequencies was adopted to ensure the maximum feed intake. Most importantly, Ravi and Devaraj (1991) have used the L-crystalline amino acids in uncoated form, which hamper the amino acid utilization by lowering its gut retentivity (Murai et al. 1982, 1984), whereas in this study, the L-crystalline amino acids were coated with casein and gelatin, which promoted the retention time of the amino acids in the gut leading to more efficient utilization of the ingested amino acids. These reasons may considerably affect the arginine requirement of *C. catla*.

Except for poor growth and feed utilization efficiency, no arginine-related deficiency signs were observed during the entire length of the feeding trial. Absence of any arginine deficiency signs in this study indicates that the diet containing minimum level of arginine (1%) was sufficient to prevent the pathological signs in this fish.

On the basis of quadratic regression analysis of growth parameters against dietary arginine concentrations, the optimum arginine requirement of fingerling *C. catla* is recommended to be 1.67% of the dry diet. Data generated during this study would be useful to formulate arginine-balanced feeds for mass culture of this fish.

Acknowledgments

The authors are grateful to the Chairman, Department of Zoology, Aligarh Muslim University, Aligarh, India, for providing necessary laboratory facilities and also to John E. Halver for supporting the Fish Nutrition Research Programme at this laboratory. We also gratefully acknowledge the financial assistance from Maulana Azad National Fellowship-University Grant Commissions for minority students for the year 2010–2011 awarded to one of us (S. Z.) and generous funding received under DST-FIST Programme, New Delhi in the priority area of Fish Nutrition.

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Fish Physiol Biochem (2013) 39:503–512
DOI 10.1007/s10695-012-9715-0

Dietary lysine requirement of fingerling *Catla catla* (Hamilton) based on growth, protein deposition, lysine retention efficiency, RNA/DNA ratio and carcass composition

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Received: 29 October 2011 / Accepted: 4 September 2012 / Published online: 12 September 2012
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Abstract A 12-week experiment was conducted to quantify dietary lysine requirement of fingerling *Catla catla* (3.65 ± 0.05 cm; 0.58 ± 0.02 g) by feeding casein–gelatine-based diets (33.0 % crude protein; 14.3 kJ/g digestible energy) with six levels of L-lysine (1.25, 1.50, 1.75, 2.00, 2.25 and 2.50 % dry diet). The experiment was conducted in eighteen 70-L indoor polyvinyl circular troughs provided with a water flow-through system (1–1.5 L/min). Live weight gain (LWG), feed conversion ratio (FCR), protein deposition (PD), lysine retention efficiency (LRE%) and RNA/DNA ratio were used as the response criteria. Second-degree polynomial regression analysis at 95 % maximum and minimum response of LWG and FCR data exhibited the lysine requirement between 1.8 and 1.9 % dry diet, corresponding to 5.5–5.7 % dietary protein. Regression analysis of PD, LRE and RNA/DNA ratio yielded the requirement between 1.7 and 1.8 % dry diet, corresponding to 5.2–5.5 % dietary protein. Since live weight gain and protein deposition are the key parameters for estimating nutrient requirement, these tools were used to recommend the lysine requirement of fingerling *C. catla* which ranges between 1.7 and 1.8 % dry diet. Data generated during this study will be useful to formulate lysine-balanced feed for intensive culture of this fish.

Keywords *Catla catla* · Fingerling · Lysine requirement · Growth · Protein deposition

Abbreviations

LWG	Live weight gain
FCR	Feed conversion ratio
PD	Protein deposition
LRE	Lysine retention efficiency
MS-222	Tricaine methanesulphonate
VSI	Viscerosomatic index
HSI	Hepatosomatic index
CF	Condition factor
CP	Crude protein
TCA	Trichloroacetic acid

Introduction

Fish have quantitative requirements for each essential amino acid. Since amino acids play important and versatile roles in protein metabolism (Wright and Fyhn 2001), dietary inclusion of amino acids should be optimum to achieve maximum growth and health benefits. Of the essential amino acids, lysine is one of the most limiting essential amino acids in ingredients used for the production of commercial fish feeds

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(Forster and Ogata 1998; Ovi and Eze 2012). Lysine is an essential amino acid present in high proportion in fish muscle tissue, involved in growth and maintenance of positive nitrogen balance and also used in 'cross-linking' protein, especially collagen (UNM 2006). Information on dietary lysine requirements of cyprinids such as mrigal *Cirrhinus mrigala* (Ahmed and Khan 2004); grass carp *Ctenopharyngodon idella* (Wang et al. 2005); rohu *Labeo rohita* (Abidi and Khan 2010); common carp, *Cyprinus carpio* (Zhou et al. 2008); and other cultivable fish species (NRC 2011) are available.

Indian major carp, *Catla catla*, is a potentially important freshwater fish species cultured in Asia, particularly in the Indian subcontinent as a component of polyculture system. It is a fast growing and an excellent food fish with high market price and adaptability to intensive culture systems. Although information on lysine requirement of the fry stage of *C. catla* is available (Ravi and Devaraj 1991), no information is available on dietary lysine requirement of fingerling *C. catla*. Therefore, this study was undertaken to work out the dietary lysine requirement of the fingerling stage of this important cultivated fish species.

Materials and methods

Experimental diet

Six isonitrogenous (33.0 % crude protein) and isocaloric (14.3 kJ/g digestible energy) amino acid test diets using casein (fat-free), gelatine and L-crystalline amino acids with graded levels of L-lysine (1.25, 1.50, 1.75, 2.00, 2.25 and 2.50 % dry diet) were prepared (Table 1). L-crystalline amino acids excluding the test amino acid lysine were used to simulate the amino acid profile of the experimental diets to that of 33.0 % whole chicken egg protein. Diets were made isonitrogenous and isocaloric by adjusting glycine and the dextrin. The levels of lysine in the amino acid test diets were fixed on the basis of information available on other two Indian major carps (Ahmed and Khan 2004; Abidi and Khan 2010). Analysed amino acid composition (% dry diet) of the experimental diets is presented in Table 2. Diets were designated as $L_{1.25}$, $L_{1.5}$, $L_{1.75}$, L_2, $L_{2.25}$ and $L_{2.5}$. To ensure maximum utilization of the limiting amino acid, the dietary protein level was fixed at 33.0 % which is slightly lower than the optimum protein requirement (35.0 %) of fingerling *C. catla* reported by Khan and Jafri (1991) and Dars et al. (2010). Method of preparation of experimental diets was the same as detailed earlier (Abidi and Khan 2007). Since L-crystalline amino acids were well coated with casein and gelatine and then bound with 10 % carboxymethyl cellulose, this provided sufficient water stability that was checked according to the method of Fagbenro and Jauncey (1995) and found to be about 98 %.

Experimental design and feeding trial

Induced bred fry *C. catla* were procured from G. B. Pant University of Agriculture and Technology, Pantnagar. These were then transported to the wet laboratory in oxygen-filled polythene bags, given a prophylactic dip in $KMnO_4$ solution (1:3,000) and stocked in indoor circular aqua-blue coloured, plastic lined (Plastic Crafts Corp, Mumbai, India) fish tanks (1.22 m × 0.91 m × 0.91 m; water volume 600 L) for 3 weeks. During this period, the fish were acclimated to a casein–gelatine-based (33.0 % CP) H-440 diet (Halver 2002) and reared to fingerling stage.

Fingerling *C. catla* (3.65 ± 0.05 cm; 0.58 ± 0.02 g) were taken from the above acclimated fish lot and stocked in triplicate groups in 70-L circular polyvinyl troughs (water volume 55 L) fitted with a continuous water flow-through (1–1.5 L/min) system at the rate of 25 fish per trough for each dietary treatment level. Fish were fed test diets in the form of dry crumbles (0.20–0.25 mm) to apparent satiation thrice a day at 08.00, 12.30 and 17.30 h. Initial and weekly weights were recorded on a top-loading balance (Precisa 120A; 0.1 mg sensitivity, Oerlikon AG, Zurich, Switzerland) after anaesthetizing the fish with tricaine methanesulphonate (MS-222; 100 μg/mL). Fish were deprived of feed on the day they were weighed. The feeding trial lasted for 12 weeks. Faecal matter was siphoned before every feeding.

Water quality parameters

Water quality parameters were recorded daily during the feeding trial (APHA 1992). The average water temperature, dissolved oxygen, free carbon dioxide, pH, total ammonia nitrogen, nitrites and total alkalinity based on daily measurements were 26.8 ± 1.6 °C, 6.8 ± 0.9 mg/L, 7.9 ± 2.1 mg/L, 7.1 ± 0.5,

Table 1 Composition of experimental diets

Ingredients (g/100 g)	Dietary lysine levels (% dry diet)					
	1.25 ($L_{1.25}$)	1.50 ($L_{1.5}$)	1.75 ($L_{1.75}$)	2.00 (L_2)	2.25 ($L_{2.25}$)	2.50 ($L_{2.5}$)
Casein[a] (fat-free)	13	13	13	13	13	13
Gelatine[b]	4.33	4.33	4.33	4.33	4.33	4.33
Dextrin	33.61	33.62	33.63	33.65	33.66	33.67
Amino acid mixture[c]	19.90	19.89	19.88	19.87	19.87	19.89
Corn oil	5	5	5	5	5	5
Cod liver oil	2	2	2	2	2	2
Mineral mix[d,f]	4	4	4	4	4	4
Vitamin mix[e,f]	3	3	3	3	3	3
α-Cellulose	5.16	5.16	5.16	5.15	5.15	5.14
Carboxymethyl cellulose	10	10	10	10	10	10
Total	100	100	100	100	100	100
Analysed crude protein	33.51	33.46	33.17	33.38	32.62	32.74
Digestible energy[g] (kJ/g, dry diet)	14.33	14.33	14.33	14.33	14.33	14.33

[a] Crude protein (76 %), [b] crude protein (96 %), [c] amino acid mixture (g/100 g dry diet); arginine 1.289, histidine 0.317, isoleucine 1.867, leucine 1.653, lysine variable, methionine 0.897, cystine 0.738, phenylalanine 0.353, tyrosine 0.819, threonine 0.895, tryptophan 0.396, valine 1.459, alanine 1.069, aspartic acid 0.079, proline 0.827, glycine variable; (Loba Chemie, India), [d] mineral mixture (g/100 g of mineral mixture), calcium biphosphate 13.57; calcium lactate 32.69; ferric citrate 02.97; magnesium sulphate 13.20; potassium phosphate (dibasic) 23.98; sodium biphosphate 08.72; sodium chloride 04.35; aluminium chloride. $6H_2O$ 0.0154; potassium iodide 0.015; cuprous chloride 0.010; magnus sulphate. H_2O 0.080; cobalt chloride. $6H_2O$ 0.100; zinc sulphate. $7H_2O$ 0.40, [e] vitamin mixture (g/100 g dry diet). choline chloride 0.500; inositol 0.200; ascorbic acid 0.100; niacin 0.075; calcium pantothenate 0.05; riboflavin 0.02; menadione 0.004; pyridoxine hydrochloride 0.005; folic acid 0.0015; biotin 0.0005; alpha-tocopherol 0.04; vitamin B_{12} 0.00001; 2 g α-cellulose, [f] Halver (2002). [g] Digestible energy was calculated on the basis of physiological fuel values 18.83, 14.64 and 35.56 kJ/g for protein, carbohydrate and fat, respectively (Jauncey 1982)

0.29 ± 0.03 mg/L, 0.07 ± 0.004 mg/L and 68.1 ± 2.41 mg/L, respectively.

Sample collection

After the end of the 12-week feeding trial, four fish from each replicate of the treatment ($n = 3 \times 4$) were anesthetized with MS-222 (tricaine methanesulphonate; 100 µg/mL). Liver and viscera of each specimen were carefully removed and weights of fish, viscera and liver were recorded to calculate viscerosomatic index (VSI), HSI and condition factor (CF).

Chemical analyses

Proximate composition of experimental diets, and initial and final whole-body carcass was estimated using standard methods (AOAC 1995) for dry matter (oven drying at 105 ± 1 °C for 22 h), crude protein (Kjeltec TecatorTM Technology 2300, Sweden), crude fat (solvent extraction with petroleum ether B.P 40–60 °C for 2–4 h using Socs Plus, SCS 4, Pelican equipments, Chennai, India) and ash (oven incineration at 650 °C for 2–4 h using muffle furnace, S.M. Scientific Instrument (p) ltd. Jindal Company, India 2–4 h). Amino acid analysis of casein, gelatine, experimental diets, initial and final whole-body fish carcass was performed by hydrolysing 0.3 mg sample in 1 mL of 6 N HCl for about 22 h. The sample thus obtained was diluted to 0.02 N HCl and injected in an automatic amino acid analyzer (Hitachi L-8800, Tokyo, Japan). Recovery hydrolysis was performed in 4 N methanesulphonic acid for the analysis of tryptophan and in performic acid for the recovery of sulphur amino acids. At the beginning of the feeding trial, 60 fish were randomly sampled, killed and pooled together. Six subsamples of a pooled sample were analysed for initial whole-body carcass composition. At the end of the experiment, 20 fishes from each replicate of dietary treatments were randomly killed with an overdose of MS-222 and pooled separately. Three subsamples of the pooled samples were analysed for final whole-body carcass composition.

Table 2 Analysed amino acid profile of the reference protein and experimental diets (% dry diet)

Amino acids	33 % whole chicken egg protein	1.25 ($L_{1.25}$)	1.50 ($L_{1.5}$)	1.75 ($L_{1.75}$)	2.00 (L_2)	2.25 ($L_{2.25}$)	2.50 ($L_{2.5}$)
EAAs							
Arginine	2.11	2.10	2.16	2.02	2.12	2.15	2.13
Histidine	0.69	0.70	0.71	0.69	0.70	0.72	0.69
Isoleucine	2.64	2.66	2.65	2.65	2.66	2.68	2.67
Leucine	3.04	3.06	3.11	3.10	3.12	3.11	3.12
Lysine	2.38	1.22	1.46	1.73	1.96	2.22	2.47
Methionine	1.36	1.37	1.35	1.38	1.37	1.36	1.37
Phenylalanine	2.08	2.12	2.13	2.11	2.14	2.10	2.11
Threonine	1.43	1.47	1.45	1.45	1.44	1.46	1.47
Tryptophan	0.50	0.53	0.52	0.53	0.53	0.51	0.52
Valine	2.41	2.43	2.44	2.46	2.41	2.42	2.44
NEAAs							
Cystine	0.79	0.81	0.83	0.80	0.82	0.81	0.83
Tyrosine	1.49	1.51	1.52	1.53	1.50	1.51	1.52
Alanine	1.90	1.89	1.91	1.88	1.92	1.87	1.88
Aspartic acid	1.14	1.16	1.15	1.14	1.13	1.16	1.17
Glycine	4.27	7.54	7.28	7.02	6.76	6.51	6.25
Proline	2.77	2.81	2.83	2.80	2.78	2.81	2.74

Determined by Hitachi L-8800 automatic amino acid analyzer

Muscle RNA/DNA ratio determination

Muscle RNA and DNA were determined by the method of Schneider (1957). After the termination of feeding trial, three fish from each replicate of the treatment group ($n = 3 \times 3$) were randomly killed with an overdose of MS-222 and white muscle tissue was removed. Three subsamples of the tissue samples for each replicate of the treatment group were taken for the determination of RNA/DNA ratio. Muscle samples (100 mg) were homogenized for 5 min in 5 % trichloroacetic acid (TCA) at 90 °C and then centrifuged at 5,000 rpm for 20 min. For the determination of RNA, 2.0 mL of distilled water and 3.0 mL of orcinol reagent were added in 1.0 mL of supernatant. The reaction mixture was kept in boiling water bath for 20 min. The greenish-blue colour thus developed was read at 660 nm. For DNA determination, in 1 mL of supernatant, 1.0 mL of distilled water and 4.0 mL of freshly prepared diphenylamine reagent were added. The reaction mixture was kept on a boiling water bath for 10 min. The blue colour developed was measured at 600 nm. Standard curves for RNA and DNA were drawn using different concentrations of yeast RNA and calf thymus DNA, respectively. The values were expressed as µg/100 mg fish muscle tissue on dry basis.

Data analyses

Growth performance of the experimental diets was measured as a function of the weight gain by calculating following parameters:

$$\text{Live weight gain} = \text{Final body weight} - \text{Initial body weight/Initial body weight}$$

$$\text{Feed conversion ratio} = \text{Dry feed intake/Wet weight gain}$$

$$\text{Feed intake (g/fish)} = \text{Total dry feed fed (g)/Total no. of fish}$$

$$\text{Lysine retention efficiency \%} = \text{Lysine gain/Lysine intake} \times 100$$

$$\text{Protein deposition} = \text{Protein gain/Protein intake}$$

$$\text{HSI \%} = \text{Liver weight (g)/Body weight (g)} \times 100$$

$$\text{VSI \%} = \text{Viscera weight (g)/Body weight (g)} \times 100$$

$$\text{CF} = \text{Body weight (g)/Body length (cm)}^3 \times 100$$

Statistical analyses

Dietary lysine requirements for fingerling *C. catla* were estimated by the second-degree polynomial regression analysis (Zeitoun et al. 1976). The equation employed was $Y = aX^2 + bX + c$. The lysine requirement for maximum growth and protein deposition is defined as the point on the abscissa representing 95 % of the value of the upper asymptote on the ordinate (Dias et al. 2003). Determination of the significance of the trend of these responses was assessed using quadratic contrasts of dietary lysine at a significance level of $P < 0.05$. All the statistical analyses were performed using Origin (version 6.1; Origin Software, San Clemente, CA, USA).

Results

Growth performance

Data related to live weight gain (LWG), feed conversion ratio (FCR), protein deposition (PD) and lysine retention efficiency (LRE%) are illustrated in Table 3. The second-degree polynomial regression analysis of the above parameters at 95 % maximum response exhibited their best values at 1.8 (Fig. 1), 1.9, 1.7 (Fig. 2) and 1.7 % lysine of the dry diet. The equations employed to establish the second-degree polynomial relationship of each variable are as under:

$$Y = -10.63X^2 + 43.11X - 32.65\left(R^2 = 0.994\right),$$
1.8 % (LWG);

$$Y = 3.07X^2 - 12.52X + 14.13\left(R^2 = 0.972\right),$$
1.9 % (FCR);

$$Y = -0.37X^2 + 1.44X - 1.11\left(R^2 = 0.935\right),$$
1.7 % (PD)

$$Y = -71.41X^2 + 279.65X - 201.92\left(R^2 = 0.918\right),$$
1.7 % (LRE)

The amino acid test diets were well accepted by the fishes of all the treatment groups and feed intake was not significantly affected by the dietary lysine concentrations (Table 3).

Whole-body composition

Whole-body composition of fish fed varying levels of dietary lysine is presented in Table 4. These parameters were affected by the varying concentrations of dietary lysine. Carcass fat content decreased linearly with the increase in lysine up to 2.0 % of the dry diet (L_2), beyond this remained almost unchanged. Moisture content exhibited the reverse trend in contrast to carcass fat. Carcass protein attained the highest value (15.6 %) for the group fed dietary lysine at 1.75 % ($L_{1.75}$). Dietary treatments did not influence carcass ash content. No mortality was recorded during the entire length of the feeding trial.

Table 3 Growth performance, feed conversion, protein deposition and lysine retention efficiency of fingerling *C. catla* fed diets with varying levels of lysine

	Dietary lysine levels (% dry diet)						
	1.25 ($L_{1.25}$)	1.50 ($L_{1.5}$)	1.75 ($L_{1.75}$)	2.00 (L_2)	2.25 ($L_{2.25}$)	2.50 ($L_{2.5}$)	Quadratic Pr > F
Average initial weight (g)	0.58 ± 0.07	0.61 ± 0.02	0.59 ± 0.01	0.59 ± 0.008	0.60 ± 0.009	0.59 ± 0.01	0.779[a]
Average final weight (g)	3.30 ± 0.02	5.46 ± 0.06	6.73 ± 0.05	7.27 ± 0.07	6.76 ± 0.11	5.77 ± 0.04	0.00008
Live weight gain	4.69 ± 0.05	7.95 ± 0.04	10.41 ± 0.09	11.32 ± 0.06	10.27 ± 0.07	8.78 ± 0.03	0.0005
Feed intake (g/fish)	9.28 ± 0.12	9.74 ± 0.11	9.33 ± 0.14	9.89 ± 0.16	9.91 ± 0.15	9.62 ± 0.12	0.432[a]
Feed conversion ratio	3.41 ± 0.11	2.01 ± 0.04	1.52 ± 0.02	1.48 ± 0.02	1.61 ± 0.04	1.86 ± 0.03	0.011
Protein deposition	0.11 ± 0.02	0.23 ± 0.01	0.32 ± 0.02	0.30 ± 0.02	0.24 ± 0.02	0.20 ± 0.01	0.018
Lysine retention efficiency %	34.71 ± 0.51	58.84 ± 0.58	76.15 ± 0.52	67.15 ± 0.51	61.21 ± 0.52	54.93 ± 0.47	0.041

Mean values of 3 replicates ± SEM

[a] Not statistically significant ($P > 0.05$)

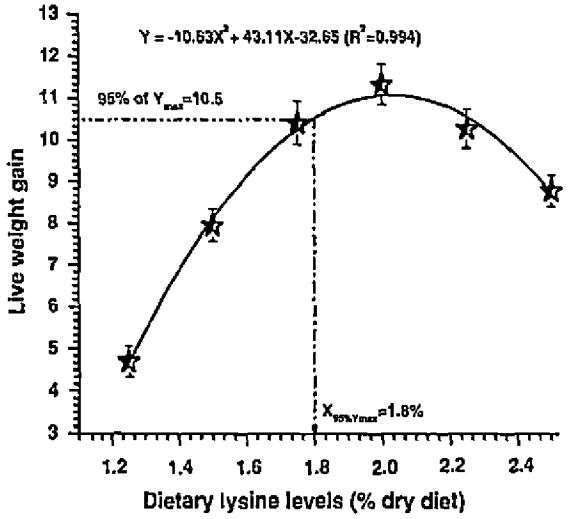

Fig. 1 Second-degree polynomial relationship of dietary lysine to live weight gain

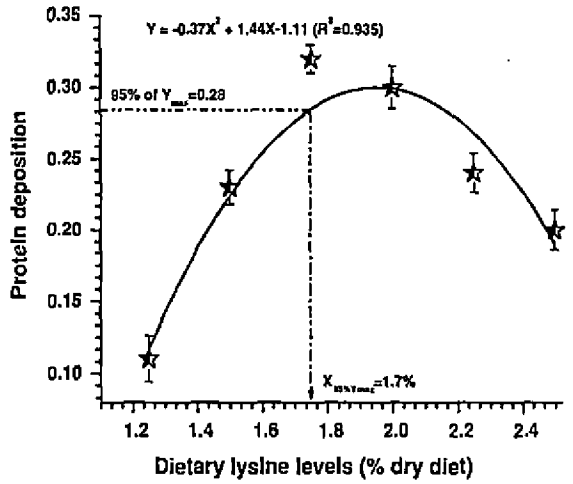

Fig. 2 Second-degree polynomial relationship of dietary lysine to protein deposition

Nucleic acid indices

Nucleic acid indices were affected by the varying levels of dietary lysine (Table 4). Muscle DNA concentration was found to decrease with the increase in dietary lysine levels up to 2.0 % (L_2) and a slight increase was noted for the groups fed higher levels of lysine at 2.25 and 2.5 % dry diet ($L_{2.25}$–$L_{2.5}$). The muscle RNA concentration was found to increase with the increased inclusion of lysine up to 1.75 % of the dry diet ($L_{1.75}$) and decreased in groups fed higher dietary lysine concentrations at 2.0, 2.25 and 2.5 % (L_2, $L_{2.25}$ and $L_{2.5}$). The data for RNA/DNA ratio were also subjected to second-degree polynomial regression analysis which at 95 % of maximum response exhibited the lysine requirement at 1.7 % of the dry diet. The second-degree polynomial regression equation employed to calculate the requirement was $Y = -5.31X^2 + 20.74X - 15.87$ ($R^2 = 0.870$).

Somatic indices

Somatic indices including HSI, VSI and CF are depicted in Table 4. Fish fed lowest level of dietary lysine ($L_{1.25}$) had highest HSI value (0.98). Viscerosomatic index was found to decrease with the increase of dietary lysine up to 2.0 % (L_2) and then increased in fish fed at higher levels (2.25 and 2.5 %) of dietary lysine ($L_{2.25}$–$L_{2.5}$). Graded levels of dietary lysine had an impact on condition factor. Lowest value of CF (0.93) was recorded for the group fed 1.25 % dietary lysine ($L_{1.25}$). However, it improved (0.93–1.58) with the increase in dietary lysine concentrations up to 2.0 % (L_2) and declined for the groups receiving higher dietary lysine concentrations ($L_{2.25}$–$L_{2.5}$).

Amino acid composition of fish carcass

The amino acid composition of fish carcass fed diets with increasing levels of lysine is given in Table 5. Lysine content of fish carcass was significantly affected by dietary lysine levels. Fish fed diet containing 1.75 % lysine ($L_{1.75}$) showed highest lysine content. Whereas lowest lysine content was recorded in group receiving 1.25 % dietary lysine ($L_{1.25}$). However, no significant ($P > 0.05$) change in concentrations of other amino acids was noted.

Second-degree polynomial regression analysis at 95 % maximum and minimum response of LWG, FCR, PD, LRE and RNA/DNA ratio against dietary lysine concentrations yielded the optimal values between 1.7 and 1.9 % of the dry diet, corresponding to 5.2–5.8 % of dietary protein.

Discussion

Since live weight gain and protein deposition are the key parameters for estimating requirement (Encarnacao et al. 2004), these parameters were used to estimate the lysine requirement of fingerling *C. catla*.

Table 4 Whole-body carcass composition (wet basis), muscle RNA/DNA ratio and somatic indices of fingerling *C. catla* fed diets with varying levels of lysine

	Dietary lysine levels (% dry diet)							
	Initial	1.25 ($L_{1.25}$)	1.50 ($L_{1.5}$)	1.75 ($L_{1.75}$)	2.00 (L_2)	2.25 ($L_{2.25}$)	2.50 ($L_{2.5}$)	Quadratic Pr > F
Moisture (%)	79.2 ± 0.3	76.3 ± 0.6	77.1 ± 0.7	77.6 ± 0.7	78.2 ± 0.6	78.1 ± 0.7	78.0 ± 0.7	0.001
Protein (%)	12.4 ± 0.06	12.5 ± 0.05	14.7 ± 0.07	15.6 ± 0.05	14.3 ± 0.06	14.0 ± 0.09	13.5 ± 0.08	0.147
Fat (%)	3.8 ± 0.11	4.9 ± 0.02	4.3 ± 0.06	3.3 ± 0.08	2.7 ± 0.04	2.6 ± 0.05	2.6 ± 0.03	0.002
Ash (%)	2.3 ± 0.08	2.3 ± 0.03	2.4 ± 0.02	2.3 ± 0.04	2.4 ± 0.03	2.4 ± 0.02	2.3 ± 0.02	0.591[a]
RNA (µg/100 mg dry weight basis)	849 ± 5	724 ± 4	978 ± 6	1,197 ± 4	1,084 ± 5	916 ± 3	827 ± 4	0.048
DNA (µg/100 mg dry weight basis)	431 ± 4	421 ± 3	314 ± 3	249 ± 2	257 ± 2	261 ± 3	266 ± 2	0.013
RNA/DNA ratio	1.9 ± 0.06	1.7 ± 0.02	3.1 ± 0.07	4.8 ± 0.04	4.2 ± 0.05	3.5 ± 0.06	3.1 ± 0.02	0.046
Viscerosomatic index %	5.9 ± 0.04	6.5 ± 0.02	5.6 ± 0.02	4.7 ± 0.05	4.4 ± 0.03	4.7 ± 0.02	5.0 ± 0.02	0.002
Hepatosomatic index %	0.8 ± 0.02	1.0 ± 0.03	0.7 ± 0.02	0.7 ± 0.02	0.6 ± 0.04	0.5 ± 0.03	0.4 ± 0.02	0.018
Condition factor	1.0 ± 0.03	0.9 ± 0.02	1.2 ± 0.05	1.4 ± 0.06	1.6 ± 0.03	1.5 ± 0.02	1.4 ± 0.08	0.002

Mean values of 3 replicates ± SEM

[a] Not statistically significant ($P > 0.05$)

The second-degree polynomial regression analysis of LWG and PD exhibited the lysine requirement of fingerling *C. catla* in the range of 1.7–1.8 % dry diet. The requirement obtained in this study is lower than that reported for common carp *C. carpio*, 2.2 %; rohu *L. rohita*, 2.3 %; tilapia *O.* spp., 2.4 %; rainbow trout *O. mykiss*, 2.4 %; Atlantic Salmon *S. salar*, 2.4 %; pacific salmon *O.* spp., 2.2 % (NRC 2011) and comparable to the requirement of *O.* spp. 1.6 %; channel catfish *I. punctatus*, 1.6 % of the dry diet (NRC 2011). The above discrepancies in lysine requirements among fishes may be attributed to differences in metabolic requirements of the species and daily protein consumption by fish, dietary formulations and feeding regimes used in the classical dose–response experiments (Cowey 1993; Fagbenro et al. 1998). In addition, use of different mathematical methods for fitting the response and estimating requirement results in different estimates of amino acid requirements (Rodehutscord and Pack 1999).

The lysine requirement of fingerling *C. catla* worked out during this study (1.7–1.8 % dry diet) is lower than the requirement reported by Ravi and Devaraj (1991) for fry stage of this fish (2.5 % dry diet). Higher amino acid requirement estimates are associated with the smaller size fish, whereas it becomes comparatively lower with the advancing stages probably as a result of the higher rates of protein synthesis and growth displayed by smaller individuals (Houlihan et al. 1986; Moltschaniwskyj and Carter 2010). This may be the reason for the differences in the lysine requirement as fish under study were in fingerling stage requiring lower dietary nutrient requirements for the metabolical and physiological activities than the fry stage of the fish in the study conducted by Ravi and Devaraj (1991). Differences in the lysine requirement of similar species may be due to differences in experimental design, available dietary energy and the composition of the specific dietary protein (Wilson 1984). Ravi and Devaraj (1991) have used the L-crystalline amino acids in unbound form hampering the amino acid utilization by lowering its gut retentivity, hence yielding to a higher requirement estimates. However, in present study, the L-crystalline amino acids were coated with casein and gelatine that have promoted the retention time of the amino acid in

Table 5 Analysed whole-body amino acid composition (g/100 g dry matter) of fingerling C. *catla* fed diets with varying levels of dietary lysine

Amino acids	1.25 ($L_{1.25}$)	1.50 ($L_{1.5}$)	1.75 ($L_{1.75}$)	2.00 (L_2)	2.25 ($L_{2.25}$)	2.50 ($L_{2.5}$)	Quadratic Pr > F
EAAs							
Arginine	4.91 ± 0.05	4.92 ± 0.09	5.11 ± 0.11	5.03 ± 0.14	5.01 ± 0.12	4.99 ± 0.14	0.249[a]
Histidine	2.10 ± 0.03	2.11 ± 0.05	2.10 ± 0.07	2.09 ± 0.06	2.06 ± 0.04	2.08 ± 0.02	0.504[a]
Isoleucine	2.21 ± 0.06	2.20 ± 0.02	2.22 ± 0.09	2.20 ± 0.04	2.21 ± 0.03	2.19 ± 0.04	0.067[a]
Leucine	4.12 ± 0.04	4.14 ± 0.07	4.15 ± 0.16	4.14 ± 0.14	4.15 ± 0.12	4.13 ± 0.15	0.053[a]
Lysine	4.25 ± 0.11	4.87 ± 0.09	5.64 ± 0.13	5.53 ± 0.11	5.47 ± 0.14	5.32 ± 0.12	0.013
Methionine + cystine	1.68 ± 0.04	1. 67 ± 0.07	1.66 ± 0.11	1.68 ± 0.13	1.67 ± 0.09	1.66 ± 0.05	0.196[a]
Phenylalanine + tyrosine	4.79 ± 0.17	4.80 ± 0.15	4.78 ± 0.14	4.81 ± 0.18	4.79 ± 0.13	4.76 ± 0.11	0.329[a]
Threonine	2.48 ± 0.04	2.50 ± 0.06	2.46 ± 0.08	2.47 ± 0.11	2.46 ± 0.13	2.49 ± 0.08	0.787[a]
Tryptophan	0.53 ± 0.02	0.51 ± 0.01	0.54 ± 0.02	0.52 ± 0.03	0.53 ± 0.02	0.54 ± 0.02	0.819[a]
Valine	2.69 ± 0.02	2.71 ± 0.05	2.68 ± 0.03	2.69 ± 0.11	2.72 ± 0.06	2.70 ± 0.08	0.461[a]
NEAAs							
Alanine	3.87 ± 0.13	3.91 ± 0.11	3.93 ± 0.09	3.88 ± 0.16	3.85 ± 0.14	3.83 ± 0.10	0.063[a]
Aspartic acid	5.57 ± 0.06	5.61 ± 0.17	5.64 ± 0.12	5.62 ± 0.14	5.59 ± 0.11	5.57 ± 0.15	0.075[a]
Glycine	4.49 ± 0.10	4.51 ± 0.08	4.59 ± 0.14	4.54 ± 0.10	4.52 ± 0.08	4.49 ± 0.12	0.006[a]
Proline	2.86 ± 0.04	2.85 ± 0.09	2.87 ± 0.07	2.84 ± 0.03	2.86 ± 0.06	2.85 ± 0.09	0.378[a]
Serine	2.30 ± 0.02	2.31 ± 0.04	2.28 ± 0.05	2.26 ± 0.08	2.27 ± 0.04	2.30 ± 0.11	0.104[a]

Determined by Hitachi L-8800 automatic amino acid analyzer

Mean values of 3 replicates ± SEM

[a] Not statistically significant ($P > 0.05$)

the gut leading to more efficient utilization of the ingested amino acids. These methodological biases may probably be the reasons for the differences obtained in the requirement estimates of the two studies.

In the present study, live weight gain of fingerling *C. catla* was found to improve quadratically up to 1.8 % dietary lysine ($Y_{95\ \%max}$) and then declined with further increase in dietary lysine levels. This trend of weight gain is in agreement with the results reported by earlier workers (Ahmed and Khan 2004; Wang et al. 2005; Mai et al. 2006; Dairiki et al. 2007; Bicudo et al. 2009; Abidi and Khan 2010; Xie et al. 2012; Furuya et al. 2012). The reduction in the growth of fish fed higher than required levels of dietary lysine may either be due to the stress caused by excess amount of amino acid in the body of the fish leading to extra energy expenditure towards deamination and excretion (Abidi and Khan 2010).

The RNA/DNA ratio has been considered to be a promising indicator of growth as it is closely related to the rate of protein synthesis (Tanaka et al. 2007). In the present study, muscle RNA/DNA ratio was found to improve quadratically up to 1.7 % lysine of the dry diet. The dietary imbalance of amino acids affects the efficiency of protein utilization. Lysine is primarily used for protein deposition (Firman 2004) which in this study was found to improve quadratically with the increased inclusion of dietary lysine up to 1.7 % ($Y_{95\ \%max}$), and a reduction was noted for the groups fed dietary lysine above this level. Carcass protein also followed the same trend. Carcass lipid responded negatively with the increasing concentrations of dietary lysine up to 2.0 % (L_2). This indicates that a better dietary amino acid balance probably prevents the selective catabolism of amino acids and consequently increases protein synthesis, while decreasing the accumulation of lipid reserves (Tantikitti and Chimsung 2001; Conceicao et al. 2003). The declining trend of carcass fat content as observed in this study may also be due to the fact that lysine is the precursor of L-carnitine (Rebouche 1992) which acts as a lipolytic factor thus contributing to a reduction in body fat deposition (Dias et al. 2001). It also facilitates

the removal of short-chain organic acids from mitochondria, thereby freeing intramitochondrial coenzyme A to participate in the β-oxidation and tricarboxylic acid cycle pathways which could avoid accumulation of lipid in fish body (Ozorio et al. 2003). The low carcass lipid content recorded for fish fed incremental levels of dietary lysine as evident in this study is in agreement with Ruchimat et al. (1997), Keshavanth and Renuka (1998), Mai et al. (2006) and Ma et al. (2007). Common adverse effects of lysine deficiency in fish are slow growth rate and poor protein utilization. These deficiency symptoms were also recorded in this study. Hence, optimizing dietary lysine is prerequisite for growth and protein deposition in fingering *C. catla*.

Second-degree polynomial regression analysis of LWG and PD data exhibited the lysine requirement between 1.7 and 1.8 % dry diet which is recommended to prepare lysine-balanced, cost-effective practical feeds for intensive aquaculture of this valuable Indian major carp species.

Acknowledgments The authors are grateful to the Chairman, Department of Zoology, Aligarh Muslim University, Aligarh, India for providing necessary laboratory facilities and also to Prof. John E. Halver for supporting the Fish Nutrition Research Programme at this laboratory. We also gratefully acknowledge the financial assistance from Maulana Azad National Fellowship-University Grant Commissions for minority students for the year 2010–2011 awarded to one of us (Seemab Zehra) and generous funding received under DST-FIST Programme, New Delhi in the priority area of Fish Nutrition.

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Aquacult Int (2013) 21:1243–1259
DOI 10.1007/s10499-013-9627-8

Dietary isoleucine requirement of fingerling catla, *Catla catla* (Hamilton), based on growth, protein productive value, isoleucine retention efficiency and carcass composition

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Received: 9 August 2012 / Accepted: 10 January 2013 / Published online: 23 January 2013
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Abstract In order to determine the dietary isoleucine requirement of fingerling catla, *Catla catla* (4.25 ± 0.15 cm, 0.61 ± 0.04 g), six isonitrogenous (33.0 % crude protein) and isocaloric (13.7 kJ/g digestible energy) amino acid test diets containing casein, gelatin and L-crystalline amino acids with graded levels of isoleucine (0.5, 0.75, 1.0, 1.25, 1.5 and 1.75 % of the dry diet) were prepared. Triplicate groups of fish were randomly stocked in eighteen 70-l indoor polyvinyl circular troughs at a density of 25 fingerling per trough provided with a water flow-through system (1–1.5 l min^{-1}). The experimental diets were fed to fish to apparent satiation at 08:00, 12:30 and 17:30 h for 12 weeks. Growth of the fish was found to increase with the incremental levels of dietary isoleucine up to 1.25 % of the dry diet. Quadratic regression analysis at 95 % maximum response of absolute weight gain (6.18 g $fish^{-1}$), protein productive value (0.32), isoleucine retention efficiency (71.91 g $fish^{-1}$), RNA/DNA ratio (4.81) and carcass protein (15.7 %) yielded the optimum isoleucine requirement in the range of 1.13–1.18 % of the dry diet, corresponding to 3.42–3.58 % of dietary protein. Data generated in this experiment would be useful to formulate isoleucine-balanced, cost-effective quality feeds for fingerling catla.

Keywords *Catla catla* · Isoleucine requirement · Growth · Protein productive value

Abbreviations

AWG	Absolute weight gain
FCR	Feed conversion ratio
PPV	Protein productive value
IRE	Isoleucine retention efficiency
MS-222	Tricaine methane sulphonate
VSI	Viscerosomatic index
HSI	Hepatosomatic index
CF	Condition factor

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CP Crude protein
TCA Trichloroacetic acid

Introduction

Successful and sustainable aquaculture depends upon the provision of nutritionally balanced, environmental friendly and economically viable practical feeds (Singh et al. 2006) that are the crucial elements in the culture of aquatic animals. Therefore, knowledge on nutrition and practical feeding of fish is essential for successful aquaculture. In aquaculture, feed is often the single largest operating cost item and can represent over 50 % of the operating costs in intensive aquaculture (El-Sayed 2004). Protein is a basic and most expensive component of fish feeds, both in terms of quantity and quality. Fish require not only a minimum level of protein but also that the essential amino acids are balanced to meet the requirements of each single one (Dacosta-Calheiros et al. 2003). The adjustment of essential amino acid as per the dietary needs of fish not only improves nutritional efficiency and feed conversion efficiency but also influences the utilization of other nutrients. If the essential amino acid requirements of fish species are known, it would be possible to meet these needs by using combinations of different cost-effective protein ingredients. The complete 10 essential amino acid requirements have been established for only a limited number of cultured fish species such as mrigal, *Cirrhinus mrigala*; Atlantic Salmon, *Salmo salar*; Common carp, *Cyprinus carpio*; rohu, *Labeo rohita*; tilapia, *Oreochromis* spp.; channel catfish, *Ictalurus punctatus*; rainbow trout, *Oncorhynchus mykiss;* and pacific salmon, *Oncorhynchus* spp. (NRC 2011).

Of the ten essential amino acids, evaluation of isoleucine requirement is of particular importance because isoleucine along with the other two branched-chain amino acids acts as nutrient regulator of protein synthesis and protein degradation. It is also involved in the insulin biosynthesis and secretion (Kimball and Jefferson 2006). In addition to this, it helps in energy production in the body and has been found to reduce twitching and tremors in animals (Braverman et al. 2003). It is the first limiting of the branched-chain amino acids in meat and bone meal as well as the first limiting of those amino acids not available in commercial feed-grade form (Wang et al. 1997).

The dietary requirements of isoleucine have been estimated for different fish species including chinook salmon, *O. tshawytscha* (Chance et al. 1964); channel catfish, *I. punctatus* (Wilson et al. 1980); rainbow trout, *O. mykiss* (Ogino 1980); Mossambique tilapia, *O. mossambicus* (Jauncey et al. 1983); rohu, *L. rohita* (Murthy and Varghese 1996; Khan and Abidi 2007); white sturgeon, *Acipenser transmontanus* (Ng and Hung 1995); red sea bream, *Pagrus major* (Forster and Ogata 1998); European sea bass, *Dicentrarchus labrax*; gilthead seabream, *Sparus aurata;* turbot, *Psetta maxima* (Kaushik 1998); Atlantic salmon, *S. salar* (Rollin 1999); mrigal, *C. mrigala* (Benakappa and Varghese 2003; Ahmed and Khan 2006); and grass carp, *Ctenopharyngodon idella* (Di et al. 2009).

Among the three Indian major carps, *Catla catla* is one of the most important, fast-growing commercially cultured fish (FAO 2006–2012). Because of its high nutritional value and good taste, it has greater consumer demand (ICLARM 2001). This fish is used as the integral component in carp polyculture system. Although information on isoleucine requirement of fry *C. catla* exists (Ravi and Devaraj 1991), no information on isoleucine requirement of fingerling stage of *C. catla* is available. Hence, this study was undertaken to determine the isoleucine requirement of fingerling *C. catla* using the dose–response method.

In the present study, absolute weight gain, feed conversion ratio, protein productive value, isoleucine retention efficiency and carcass protein were used as growth indicators to estimate the isoleucine requirement of this fish. Relevance of RNA and DNA data in growth and condition assessment of fish has been documented by a number of authors (Mustafa 1977; Bulow 1987; Abidi and Khan 2009). Considering the significance of RNA/DNA ratio as an index of protein synthetic machinery in cells and therefore as a sensitive indicator of fish growth in response to nutritional status (Clemmesen 1994), this parameter was also employed, in addition to growth parameters, to quantify the dietary isoleucine requirement.

Materials and methods

Experimental diets

Six isonitrogenous (33.0 % crude protein) and isocaloric (13.7 kJ g^{-1} digestible energy) amino acid test diets using casein (fat-free), gelatin and L-crystalline amino acids with graded levels of isoleucine (0.5, 0.75, 1.0, 1.25, 1.5 and 1.75 % of dry diet) were prepared (Tables 1, 2). The levels of isoleucine in the amino acid test diets were fixed on the basis of information available on other two Indian major carps (Murthy and Varghese 1996; Khan and Abidi 2007; Benakappa and Varghese 2003; Ahmed and Khan 2006). The dietary protein level was fixed at 33 %, which is lower than the optimum protein requirement (35 %) of fingerling *C. catla* (Khan and Jafri 1991; Dars et al. 2010). This reduction was made to ensure maximum utilization of the limiting amino acid from the diet (Wilson 2002). The amino acid composition of the experimental diets simulated that of 33 % whole chicken egg protein excluding the test amino acid isoleucine. Analysed amino acid composition of 33 % whole chicken egg protein reflected the arginine, histidine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, cystine, tyrosine, alanine, aspartic acid, glutamic acid, proline and serine contents to be 2.11, 0.69, 3.04, 2.38, 1.36, 2.08, 1.43, 0.5, 2.41, 0.79, 1.49, 1.9, 1.14, 2.34, 2.77 and 0.57 % of the dry matter, respectively. The isoleucine content contributed by the casein and gelatin in experimental diets was 0.45 and 0.03 % of dry diet, respectively. The amount of isoleucine was increased at the expense of glycine, on nitrogen to nitrogen to attain the intended concentrations of dietary isoleucine in the amino acid test diets. Diets were made isonitrogenous and isocaloric by adjusting the levels of glycine and dextrin. The diets were designated as $I_{0.5}$, $I_{0.75}$, I_1, $I_{1.25}$, $I_{1.5}$ and $I_{1.75}$. Method of preparation of experimental diets was the same as detailed earlier (Abidi and Khan 2007). The L-crystalline amino acids were coated with casein and gelatin, and then 10 % of carboxymethyl cellulose was added to provide sufficient water stability which was checked and found to be 96 %. Water stability of the diet was estimated by the method adopted by Fagbenro and Jauncey (1995). In addition to provide sufficient water stability, coating of L-crystalline amino acids also reduces the absorption rate of the amino acids (Cho et al. 1992) and leaching (Alam et al. 2004). We adopted this approach as a safety measure to prevent the leaching of crystalline amino acid from the test diets.

Experimental design and feeding trial

Fries of *C. catla* were procured from G.B. Pant University of Agriculture and Technology, Pantnagar, transported to the wet laboratory in oxygen-filled polythene bags, given

Table 1 Composition of the experimental diets

Ingredients (g 100 g^{-1})	Dietary isoleucine levels (% dry diet)					
	0.5 ($I_{0.5}$)	0.75 ($I_{0.75}$)	1.0 (I_1)	1.25 ($I_{1.25}$)	1.5 ($I_{1.5}$)	1.75 ($I_{1.75}$)
Casein[a] (fat-free)	8	8	8	8	8	8
Gelatin[b]	2.67	2.67	2.67	2.67	2.67	2.67
Dextrin	34.75	34.59	34.43	34.26	34.10	33.94
Crystalline amino acid mixture[c]	25.28	25.38	25.49	25.60	25.70	25.81
Corn oil	5	5	5	5	5	5
Cod liver oil	2	2	2	2	2	2
Mineral mix[d,f]	4	4	4	4	4	4
Vitamin mix[e,f]	3	3	3	3	3	3
α-Cellulose	5.31	5.36	4.42	5.47	5.53	5.58
Carboxymethyl cellulose	10	10	10	10	10	10
Total	100	100	100	100	100	100
Added crystalline isoleucine	0.02	0.27	0.52	0.77	1.02	1.27
Total analysed isoleucine	0.51	0.73	0.98	1.23	1.47	1.73
Analysed crude protein	33.72	33.64	33.17	32.61	32.59	33.28
Digestible energy[g] (kJ g^{-1}, dry diet)	13.79	13.76	13.74	13.72	13.69	13.67

[a] Crude protein (76 %); [b] crude protein (96 %); [c] amino acid mixture (g 100 g^{-1}): arginine 1.605, histidine 0.461, isoleucine variable, leucine 2.187, lysine 1.619, methionine 1.075, cystine 0.758, phenylalanine 1.632, tyrosine 1.077, threonine 1.101, tryptophan 0.436, valine 1.825, alanine 1.388, aspartic acid 0.486, glutamic acid 0.393, proline 1.573, serine 0.189, glycine variable; [d] mineral mixture (g 100 g^{-1}): calcium biphosphate 13.57, calcium lactate 32.69, ferric citrate 02.97, magnesium sulphate 13.20, potassium phosphate (dibasic) 23.98, sodium biphosphate 08.72, sodium chloride 04.35, aluminium chloride. $6H_2O$ 0.0154, potassium iodide 0.015, cuprous chloride 0.010, manganous sulphate. H_2O 0.080, cobalt chloride. $6H_2O$ 0.100, zinc sulphate. $7H_2O$ 0.40; [e] vitamin mixture (1 g vitamin mix + 2 g α-cellulose): choline chloride 0.500, inositol 0.200, ascorbic acid 0.100, niacin 0.075, calcium pantothenate 0.05, riboflavin 0.02, menadione 0.004, pyridoxine hydrochloride 0.005, thiamine hydrochloride 0.005, folic acid 0.0015, biotin 0.0005, alpha-tocopherol 0.04, vitamin B_{12} 0.00001 [f] Halver (2002); [g] digestible energy was calculated on the basis of physiological fuel values 18.83, 14.64 and 35.56 kJ g^{-1} for protein, carbohydrate and fat, respectively (Jauncey 1982)

a prophylactic dip in $KMnO_4$ solution (1:3,000) before stocking in indoor circular aqua-blue-coloured, plastic lined (Plastic Crafts Corp, Mumbai, India) fish tanks (1.22 m in diameter and 0.91 m in height; water volume 600 l) for 3 weeks. During this period, the fish were acclimated on a casein–gelatin (33 % CP)-based H-440 diet (Halver 2002) and reared to fingerling stage.

Fingerling *C. catla* (4.25 ± 0.15 cm, 0.61 ± 0.04 g) were taken from the above acclimated fish lot and stocked in triplicate groups at the rate of 25 fish per trough for each dietary treatment level in 70-l circular polyvinyl troughs (water volume 55 l) fitted with a continuous water flow-through (1–1.5 l min^{-1}) system. Fish were fed the test diets in the form of dry crumbles (0.20–0.25 mm) to apparent satiation thrice daily at 08:00, 12:30 and 17:30 h. Initial and weekly weights were recorded on a top-loading balance (Precisa 120A; 0.1 mg sensitivity, Oerlikon AG, Zurich, Switzerland). Fish were deprived of feed on the day they were weighed. The feeding trial lasted for 12 weeks. Faecal matter was syphoned before every feeding. Water-quality indices were recorded following standard methods

Table 2 Analysed amino acid composition of the experimental diets (%) for fingerling *C. catla*

Amino acid	0.5 ($I_{0.5}$)	0.75 ($I_{0.75}$)	1.0 (I_1)	1.25 ($I_{1.25}$)	1.5 ($I_{1.5}$)	1.75 ($I_{1.75}$)
EAAs						
Arginine	2.10	2.16	2.02	2.12	2.15	2.13
Histidine	0.70	0.71	0.69	0.70	0.72	0.69
Isoleucine	0.51	0.73	0.98	1.23	1.47	1.73
Leucine	3.06	3.11	3.10	3.12	3.11	3.12
Lysine	2.38	2.34	2.32	2.41	2.39	2.40
Methionine	1.38	1.37	1.34	1.33	1.39	1.38
Phenylalanine	2.05	2.11	2.10	2.12	2.11	2.13
Threonine	1.45	1.42	1.46	1.48	1.41	1.44
Tryptophan	0.51	0.53	0.51	0.52	0.54	0.55
Valine	2.43	2.44	2.46	2.41	2.42	2.44
NEAAs						
Cystine	0.78	0.81	0.82	0.84	0.82	0.85
Tyrosine	1.50	1.48	1.47	1.44	1.46	1.54
Alanine	1.93	1.96	1.90	1.87	1.86	1.84
Aspartic acid	1.12	1.15	1.16	1.13	1.12	1.17
Glutamic acid	2.31	2.36	2.32	2.36	2.35	2.38
Glycine	7.62	7.50	7.36	7.25	7.11	6.94
Proline	2.83	2.82	2.81	2.79	2.80	2.78
Serine	0.57	0.58	0.59	0.55	0.59	0.54

Determined by Hitachi L-8800 automatic amino acid analyser

(APHA 1992). The range of water temperature, dissolved oxygen, free carbon dioxide, pH, total ammonia nitrogen, nitrites and total alkalinity, based on daily measurements, were 27.1–28.4 °C, 6.4–7.1, 5.8–10.1, 7.2–7.6, 0.27–0.34, 0.03–0.07 and 68.4–81.6 mg l^{-1}, respectively.

Sample collection

At the beginning of the feeding trial, 60 fish were randomly sampled, killed and pooled. Six subsamples of the pooled sample were analysed for initial carcass composition. At the end of the experiment, 15 fishes from each replicate of all the dietary isoleucine treatments were randomly killed and pooled. Three subsamples of the pooled samples were analysed for final carcass composition. Another five fish from each replicate ($n = 3 \times 5$) were anesthetized with MS-222 (tricaine methane sulphonate; 100 μg ml^{-1}), and liver and viscera of each specimen were carefully removed. Weight of fish, viscera and liver were recorded to calculate viscerosomatic index (VSI), hepatosomatic index (HSI) and condition factor (CF). After taking the weight of fish, viscera and liver, muscle tissue was removed from these fish. Muscle samples (100 mg) were homogenized for 5 min in 5 % of trichloroacetic acid (TCA) at 90 °C and then centrifuged at 5,000 rpm for 20 min and the supernatant removed. Three subsamples of each tissue sample were taken for the determination of RNA/DNA ratio.

Evaluation of growth parameters

Growth performance of the fish in response to the experimental diets was measured as a function of the weight gain by calculating the following parameters:

$$\text{Absolute weight gain } (\text{g fish}^{-1}) = \text{Final body weight} - \text{Initial body weight}$$

$$\text{Feed conversion ratio} = \text{Dry feed intake/Wet weight gain}$$

$$\text{Feed intake } (\text{g fish}^{-1}) = \text{Total dry feed intake (g)/Total no. of fish}$$

$$\text{Protein productive value} = \text{Protein gain/protein intake}$$

$$\text{Isoleucine retention efficiency (\%)} = \text{Isoleucine gain/Isoleucine intake} \times 100$$

$$\text{Hepatosomatic index (\%)} = \text{Liver weight (g)/Body weight (g)} \times 100$$

$$\text{Viscerosomatic index (\%)} = \text{Viscera weight (g)/Body weight (g)} \times 100$$

$$\text{Condition factor} = \text{Body weight (g)/Total body length (cm)}^3 \times 100$$

Chemical analyses

Proximate composition of experimental diets, and initial and final carcass were analysed using standard methods (AOAC 1995) for dry matter (oven drying at 105 ± 1 °C for 22 h), crude protein (Kjeldhal nitrogen × 6.25 using Kjeltec TecatorTM Technology 2300, Sweden), crude fat (solvent extraction with petroleum ether B.P 40–60 °C for 2–4 h using Socs Plus, SCS 4, Pelican equipments, Chennai, India) and ash (oven incineration at 650 °C for 2–4 h using muffle furnace, S.M. Scientific Instrument (p) ltd. Jindal Company, India). Amino acid analysis of casein, gelatin, experimental diets, initial and final fish carcass were performed by hydrolysing 0.3 mg sample in 1 ml of 6 N HCl for about 22 h. The sample thus obtained was diluted in 0.02 N HCl and injected in an Automatic Amino Acid Analyzer (Hitachi L-8800, Tokyo, Japan). Recovery hydrolysis was performed in 4 N methanesulfonic acid for the analysis of tryptophan and in performic acid for the recovery of sulphur amino acids.

Determination of muscle RNA and DNA

Muscle RNA and DNA were determined according to Schneider (1957). For the determination of RNA, 2.0 ml of distilled water and 3.0 ml of orcinol reagent were added to 1.0 ml of supernatant. The reaction mixture was kept in boiling water bath for 20 min. The greenish-blue colour thus developed was read at 660 nm in a spectrophotometer (Genesis 10-UV, Thermo Spectronic, Madison, USA). For DNA determination, 1.0 ml of distilled water and 4.0 ml of freshly prepared diphenylamine reagent were added to 1.0 ml of the supernatant. The reaction mixture was kept on a boiling water bath for 10 min. The blue colour developed was measured at 600 nm. Standard curves for RNA and DNA were drawn using different concentrations of yeast RNA and calf thymus DNA, respectively.

Statistical analyses

All growth data were subjected to one-way analysis of variance (Sokal and Rohlf 1981). Differences among treatment means were determined by Tukey's honestly significant difference (HSD) test at a $P < 0.05$ level of significance. Dietary isoleucine requirement of

fingerling *C. catla* was estimated by fitting a polynomial quadratic regression to the dose–growth responses relationship (Shearer 2000). The equation employed was $Y = aX^2 + bX + c$. The requirement was estimated to be that level eliciting 95 % of maximum response (Rodehutscord et al. 1997; Dias et al. 2003; Gaylord et al. 2005). Data analyses were performed using the statistical software Origin (version 6.1; Origin Software, San Clemente, CA, USA).

Results

Absolute weight gain (AWG), feed conversion ratio (FCR), protein productive value (PPV), isoleucine retention efficiency (IRE) and RNA/DNA ratio of fingerling *C. catla* fed diets with graded levels of isoleucine over the 12-week feeding trial were found to increase significantly with the increase in dietary isoleucine concentrations up to 1.25 % (Table 3). The best values of AWG (6.52 g $fish^{-1}$), FCR (1.45), PPV (0.35), IRE (74.13 %) and RNA/DNA ratio (5.20) were observed in fish fed diet $I_{1.25}$. No significant differences ($P > 0.05$) in growth parameters were recorded in fish fed 1.5 % of isoleucine diet. However, further increase in dietary isoleucine concentration (1.75 %) led to significant fall ($P < 0.05$) in growth parameters. Feed intake did not differ significantly among treatment groups. No mortality was recorded in all the treatment groups.

Dietary isoleucine concentrations had an impact on carcass composition of fingerling *C. catla* (Table 4). Moisture content showed a reverse trend to that of the dietary isoleucine concentrations up to 1.25 % ($I_{1.25}$). Carcass protein content was found to improve quadratically ($P < 0.05$) with the increase in dietary isoleucine concentrations up to 1.25 % with the highest value (16.54 %) at this level ($I_{1.25}$). A further inclusion of dietary isoleucine ($I_{1.5}$–$I_{1.75}$) resulted in a slight reduction ($P > 0.05$) in carcass protein. Carcass fat showed a decreasing trend up to 1.25 % of dietary isoleucine ($I_{1.25}$) beyond that no significant change was recorded. Carcass ash content remained almost the same except those fed diet $I_{0.5}$ and $I_{0.75}$.

Somatic indices including HSI, VSI and CF are illustrated in Table 4. The HSI did not show marked variations with the dietary isoleucine levels except for the fish receiving diet $I_{0.5}$. Fish fed the lowest level of dietary isoleucine ($I_{0.5}$) had the highest value of HSI. A negative correlation in VSI with the increasing concentrations of dietary isoleucine was noted up to 1.25 % ($I_{1.25}$). It was found to increase with the further increase in dietary isoleucine ($I_{1.5}$–$I_{1.75}$). The CF improved with the increase in dietary isoleucine and was found to be highest at 1.25 %. Fish fed higher levels of dietary isoleucine at 1.5 ($I_{1.5}$) and 1.75 % ($I_{1.75}$) showed significant decline in CF.

In order to find precise isoleucine requirement, AWG, PPV, IRE, RNA/DNA ratio and carcass protein data were subjected to quadratic regression analysis which at 95 % maximum response exhibited the optimum dietary isoleucine requirement to range between 1.13 and 1.18 % of dry diets which is equivalent to 3.42–3.58 % of dietary protein. The quadratic equations employed for AWG (Fig. 1), PPV (Fig. 2), IRE (Fig. 3), RNA/DNA ratio (Fig. 4) and carcass protein (Fig. 5) are depicted in the respective figures.

Discussion

Dietary isoleucine requirement of fingerling *C. catla* (3.42–3.58 % dietary protein) worked out in this experiment is found to be higher than that reported for chinook salmon,

Table 3 Growth performance of fingerling *C. catla* fed diets containing varying levels of isoleucine

	Dietary isoleucine levels (% dry diet)						Quadratic $P > F$
	0.5 ($I_{0.5}$)	0.75 ($I_{0.75}$)	1.0 (I_1)	1.25 ($I_{1.25}$)	1.5 ($I_{1.5}$)	1.75 ($I_{1.75}$)	
Average initial weight (g)	0.61 ± 0.02^a	0.61 ± 0.05^a	0.62 ± 0.03^a	0.61 ± 0.05^a	0.61 ± 0.02^a	0.60 ± 0.08^a	0.133
Average final weight (g)	3.07 ± 0.05^d	5.29 ± 0.04^c	6.07 ± 0.05^b	7.13 ± 0.03^a	7.11 ± 0.02^a	6.16 ± 0.04^b	0.002
Absolute weight gain (g fish^{-1})	2.46 ± 0.04^d	4.68 ± 0.07^c	5.45 ± 0.03^b	6.52 ± 0.04^a	6.50 ± 0.02^a	5.56 ± 0.05^b	0.001
Feed conversion ratio	3.92 ± 0.02^a	2.11 ± 0.04^b	1.79 ± 0.02^c	1.45 ± 0.02^d	1.47 ± 0.01^d	1.70 ± 0.02^c	0.013
Feed intake (g fish^{-1})	9.64 ± 0.23^a	9.87 ± 0.17^a	9.76 ± 0.14^a	9.45 ± 0.16^a	9.56 ± 0.14^a	9.45 ± 0.15^a	0.320
Protein productive value	0.12 ± 0.01^e	0.19 ± 0.02^d	0.27 ± 0.01^c	0.35 ± 0.01^a	0.34 ± 0.02^a	0.29 ± 0.03^b	0.0097
Isoleucine retention efficiency (%)	32.61 ± 0.61^e	58.48 ± 0.54^d	65.24 ± 0.73^c	74.13 ± 0.51^a	73.98 ± 0.64^a	67.85 ± 0.58^b	0.004
RNA/DNA ratio	2.29 ± 0.01^e	3.38 ± 0.02^d	4.21 ± 0.02^c	5.20 ± 0.04^a	5.11 ± 0.03^a	4.68 ± 0.02^b	0.0036

Mean values of 3 replicates ± SEM. Mean values sharing the same superscripts in the same row are insignificantly different ($P > 0.05$)

Table 4 Carcass composition (%wet basis) and somatic indices of fingerling *C. catla* fed diets containing varying levels of isoleucine

	Dietary isoleucine levels (% dry diet)							Quadratic $P > F$
	Initial	0.5 ($I_{0.5}$)	0.75 ($I_{0.75}$)	1.0 (I_1)	1.25 ($I_{1.25}$)	1.5 ($I_{1.5}$)	1.75 ($I_{1.75}$)	
Moisture (%)	79.81 ± 0.54	74.19 ± 0.52^d	75.51 ± 0.41^c	76.28 ± 0.46^b	77.14 ± 0.39^a	77.81 ± 0.31^a	77.43 ± 0.42^a	0.0019
Protein (%)	12.27 ± 0.13	12.74 ± 0.05^d	13.62 ± 0.07^c	15.43 ± 0.06^b	16.54 ± 0.11^a	16.48 ± 0.09^a	16.26 ± 0.08^a	0.0094
Fat (%)	3.26 ± 0.11	4.97 ± 0.02^a	4.12 ± 0.04^b	3.05 ± 0.03^c	2.69 ± 0.05^d	2.71 ± 0.02^d	2.76 ± 0.05^d	0.0017
Ash (%)	2.39 ± 0.04	2.48 ± 0.03^a	2.37 ± 0.02^b	2.28 ± 0.02^c	2.26 ± 0.02^c	2.27 ± 0.03^c	2.29 ± 0.02^c	0.0011
Hepatosomatic index (%)	0.84 ± 0.06	1.21 ± 0.05^a	0.91 ± 0.03^b	0.89 ± 0.02^b	0.87 ± 0.02^b	0.85 ± 0.04^b	0.85 ± 0.03^b	0.0491
Viscerosomatic index (%)	6.32 ± 0.11	6.51 ± 0.08^a	5.74 ± 0.11^b	5.11 ± 0.14^c	4.27 ± 0.08^e	4.75 ± 0.06^d	5.38 ± 0.02^c	0.02
Condition factor	0.91 ± 0.04	0.97 ± 0.04^c	1.23 ± 0.07^d	1.42 ± 0.09^e	1.67 ± 0.11^a	1.51 ± 0.08^b	1.44 ± 0.06^c	0.013

Mean values of 3 replicates ± SEM. Mean values sharing the same superscripts in the same row are insignificantly different ($P > 0.05$)

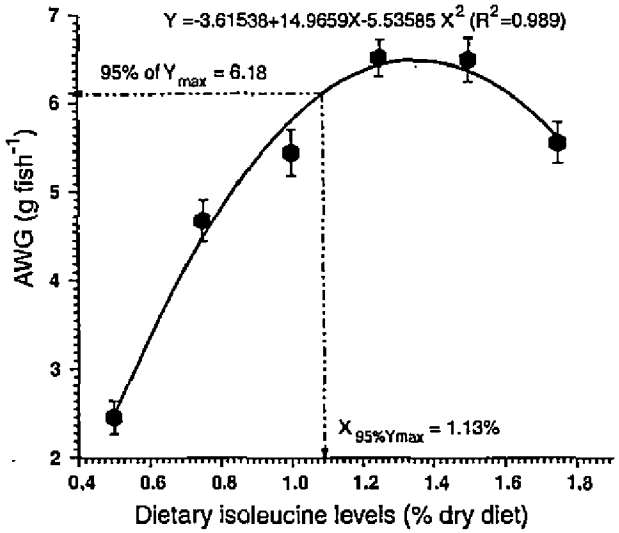

Fig. 1 Quadratic relationship of dietary isoleucine to absolute weight gain

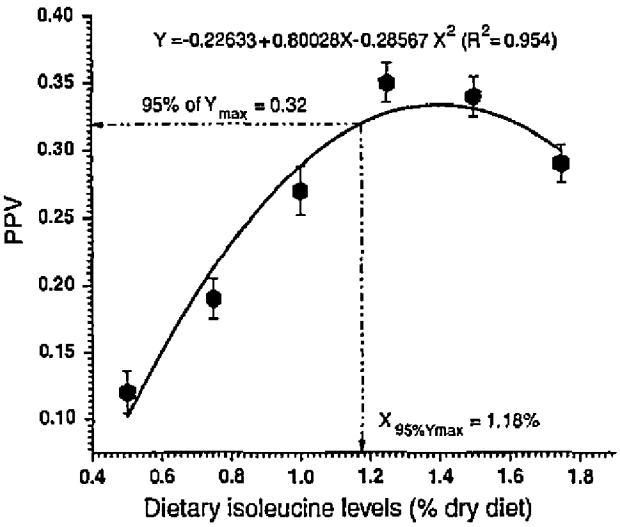

Fig. 2 Quadratic relationship of dietary isoleucine to protein productive value

O. tshawytscha 2.2 % (Chance et al. 1964); common carp, *C. carpio* 2.5 % (Nose 1979); channel catfish, *I. punctatus* 2.6 % (Wilson et al. 1980); rainbow trout, *O. mykiss* 2.4 % (Ogino 1980); Nile tilapia, *O. niloticus* 3.1 % (Santiago and Lovell 1988); chum salmon, *O. keta* 2.4 % (Akiyama and Arai 1993); coho salmon, *O. kisutch* 1.2 % (Arai and Ogata 1993); white sturgeon, *A. transmontanus* 3.0 % (Ng and Hung 1995); Japanese flounder, *Paralichthys olivaceus* 2.0 %; red sea bream, *P. major* 2.2 % (Forster and Ogata 1998); turbot, *P. maxima* 2.60 % (Kaushik 1998); Atlantic salmon, *S. salar* 3.2 % (Rollin 1999); mrigal, *C. mrigala* 3.12–3.15 % (Benakappa and Varghese 2003, Ahmed and Khan 2006) but lower than the requirement reported for milkfish, *Chanos chanos* 4.0 % (Borlongan and

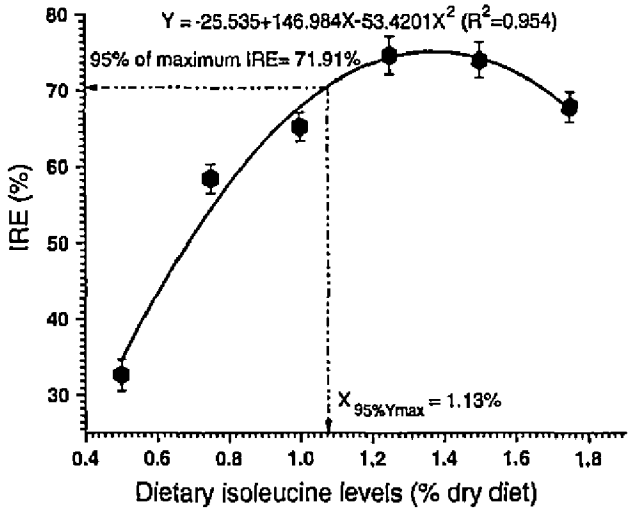

Fig. 3 Quadratic relationship of dietary isoleucine to isoleucine retention efficiency

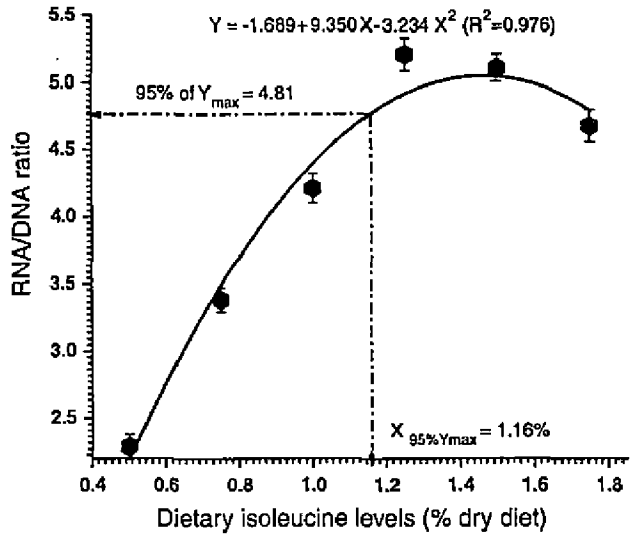

Fig. 4 Quadratic relationship of dietary isoleucine to RNA/DNA ratio

Coloso 1993); grass carp, *Ctenopharyngodon idella* 4.0–4.23 % (Di et al. 2009) and comparable to the requirement of rohu, *L. rohita* 3.75 % (Khan and Abidi 2007) and tilapia 3.45 % of dietary protein (NRC 2011). The above discrepancies in amino acid requirements of fish may be affected by fish size and age, adequate levels of other nutrients, flow rate, stock density, and the environmental and culture conditions adopted by different laboratories (Cowey and Luquet 1983; Kim et al. 1992; Forster and Ogata 1998; Luzzana et al. 1998; Abidi and Khan 2009). Nutrient and energy digestibility, amino acid profile and energy content may also alter the amino acid requirements (Simmons et al. 1999; De Silva et al. 2000).

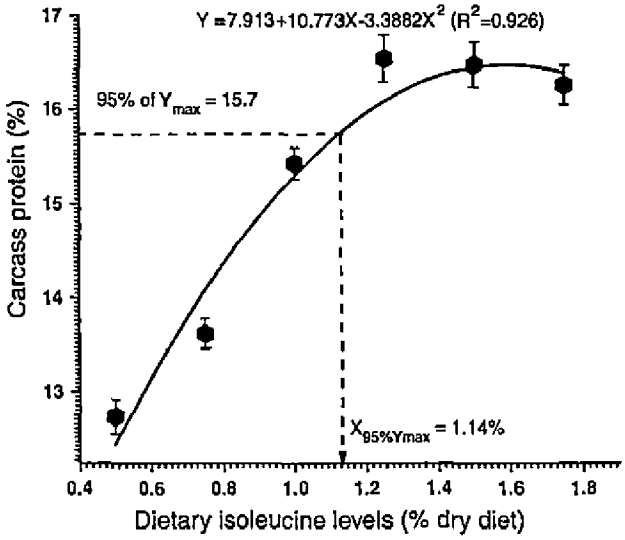

Fig. 5 Quadratic relationship of dietary isoleucine to carcass protein

Isoleucine requirement of fingering *C. catla* in the present study (3.42–3.58 % dietary protein) is higher than the requirement reported by Ravi and Devaraj (1991) for fry stage of this fish (2.35 % dietary protein). Ravi and Devaraj (1991) reported the isoleucine requirement by subjecting the weight gain data to broken-line regression analysis that has been reported to underestimate the requirement (Shearer 2000). However, in this study, the isoleucine requirement is worked out on the basis of quadratic regression analysis indicating high R^2 values (Figs. 1, 2, 3, 4, 5). Adoption of these statistical models may influence the estimate of the isoleucine requirements in both the studies. Moreover, the above low isoleucine requirement reported by Ravi and Devaraj (1991) is based on subjecting the weight gain data to regression analysis. Whereas in this study, the isoleucine requirement is based on subjecting the sensitive growth parameters such as protein productive value, isoleucine retention efficiency, RNA/DNA ratio and carcass protein to quadratic regression analysis in addition to growth. In addition to these, the different dietary protein levels adopted in this study (33 % dry diet) and that reported by Ravi and Devaraj (1991; 40 % dry diet) might also be responsible for the variation in the isoleucine requirements of *C. catla*. A huge difference in isoleucine requirement estimates in both the studies is evident when requirements are expressed as % protein, and on the other hand, the same difference become minor indeed when requirements are expressed as % dry diet (1.13–1.18 vs. 0.94). Hence, a lower dietary protein level in this study relative to that used by Ravi and Devaraj may also and simply explain higher isoleucine requirement estimates.

Determination of essential amino acid requirements in studies using the dose–response approach requires addition of large amount of crystalline amino acids to obtain graded-level of test amino acid. The incorporation, however, often leads to depressed growth performance and biased amino acid requirement estimates because of crystalline amino acid leaching, different absorption kinetics of crystalline amino acids and poor diet palatability (Ambardekar et al. 2009). Coating of crystalline amino acids can reduce the solubility and non-synchronous absorption of free amino acid relative to the protein-bound ones (Segovia-Quintero and Reigh 2004; Zhou et al. 2012). Ravi and Devaraj (1991) have used the L-crystalline amino acids in unbound form, which might have hampered the amino

acid utilization because of reduced gut retention. However, in this study, the L-crystalline amino acids in the experimental diets were coated by casein and gelatin which increases the retention time of the amino acid in the gut leading to more efficient utilization of the ingested amino acids.

In this study, absolute weight gain was found to improve with the increase in concentration of dietary isoleucine up to 1.25 % ($I_{1.25}$). Further inclusion of isoleucine ($I_{1.5}$–$I_{1.75}$) in diets led to reduction in growth of the fish. Similarly, protein productive value was found to improve with the increased inclusion of dietary isoleucine from 0.5 to 1.25 % ($I_{0.5}$–$I_{1.25}$). However, a reduction in protein productive value at higher levels of dietary isoleucine ($I_{1.5}$–$I_{1.75}$) was recorded. This reduction in growth performance at higher levels of dietary isoleucine may be attributed to amino acid toxicity. It has also been reported that excessive levels of amino acids may become toxic and have an adverse effect on growth because the disproportionate amount of one amino acid affects the absorption and utilization of other amino acids (Murthy and Varghese 1996). The major proportion of the limiting amino acids is used for protein synthesis, while amino acid in excess will be more available for oxidation (Gahl et al. 1996), which may be the cause of growth depression at higher levels of dietary isoleucine.

Nutritional and metabolic interactions among the branched-chain amino acids isoleucine, leucine and valine have been reported for various warm blooded animals, including man (Hambraeus et al. 1976); poultry (De'Mello and Lewis 1971; Smith and Austic 1978); rat (Harper et al. 1970); and the pig (Oestemer et al. 1973). Data on interactions among branched-chain amino acids in fish are not clear-cut and are inconsistent among species (Yamamoto et al. 2004). Chance et al. (1964) reported that isoleucine requirement in chinook salmon was influenced by dietary leucine and that excess dietary isoleucine reduced growth rates when leucine was deficient. In this study, interactions of isoleucine with the other two branched-chain amino acids were not studied.

The RNA/DNA ratio was found to increase with the increased inclusion of dietary isoleucine up to 1.25 % ($I_{1.25}$). Further inclusion of isoleucine ($I_{1.5}$–$I_{1.75}$) resulted in declining RNA/DNA ratio in muscle tissue. Somatic indices including HSI, VSI and CF were also affected by varying concentrations of isoleucine. The HSI value was higher for the group fed the lowest level of dietary isoleucine ($I_{0.5}$), which may probably be due to excess accumulation of fat in liver. Farhat and Khan (2011) have also reported that the higher value of HSI in fish fed at higher levels of dietary lysine might be due to deposition of fat in liver. A negative correlation in VSI with the increasing concentrations of dietary isoleucine was noted up to 1.25 % ($I_{1.25}$). Further inclusion of dietary isoleucine at 1.5 ($I_{1.5}$) and 1.75 % ($I_{1.75}$) resulted in a slight increment of VSI values. The CF was also found to improve significantly with the increase in the levels of dietary isoleucine and found to be the highest at 1.25 % isoleucine ($I_{1.25}$) in dry diet. Further increase in dietary isoleucine ($I_{1.5}$–$I_{1.75}$) showed a significant drop in CF. Di et al. (2009) also reported the same pattern of RNA/DNA ratio, VSI and CF in grass carp fed graded levels of isoleucine in the diet.

Deficiency of isoleucine causes loss of weight and poor feed conversion in milk fish (Borlongan and Coloso 1993); rohu (Murthy and Varghese 1996; Khan and Abidi 2007); and mrigal (Ahmed and Khan 2006). However, no study has addressed the other pathological signs such as spinal deformities, bilateral cataracts and caudal fin erosion due to deficiency of isoleucine in fish. Except poor growth and feed utilization efficiency, no other diet-related pathological signs were recorded in this study. All fish were found to be in good health condition.

It has been reported that the farmed *C. catla* deposited significantly higher lipid contents in liver (Hassan et al. 2010). Since the level of fat deposition affects carcass quality, the

mobilization of these lipid reserves is essential to improve the carcass quality. As the carcass fat content of this fish was found in the range of 3.26–5.48 % as reported in this study and by earlier workers (Seenappa and Devaraj 1995; Zehra and Khan 2012), this fish is considered to be medium fatty fish as per the classification of Lambertsen (1978). Fatty fish are prone to oxidation. Oxidation of lipids not only produces rancid odours and flavours, but can also decrease nutritional quality and safety by the formation of secondary products (Frankel 1998; Hsieh and Kinsella 1989). Dietary isoleucine contributes to the improvement of the carcass quality as it increases the activity of uncoupling proteins in muscle cells which increased fat burning. Supplementing isoleucine might be a simple way to speed metabolic rate and lose body fat (Di et al. 2009; Nishimura et al. 2010). In this study, carcass fat was found to decrease linearly with the increasing concentrations of dietary isoleucine up to 1.25 % ($I_{1.25}$). However, carcass protein showed a positive trend with the increasing levels of dietary isoleucine up to 1.25 % ($I_{1.25}$). A similar trend of carcass fat and carcass protein has also been reported by Di et al. (2009) in grass carp fed graded levels of dietary isoleucine.

In conclusion, the quadratic regression analysis of growth parameters at 95 % maximum response exhibited the optimum dietary isoleucine requirement between 1.13 and 1.18 % of dry diets, corresponding to 3.42–3.58 % of dietary protein, and hence is recommended for fingerling *C. catla*. Data generated by the present study would be useful in formulating isoleucine-balanced feeds for the intensive culture of fingerling *C. catla*.

Acknowledgments The authors are grateful to the Chairman, Department of Zoology, Aligarh Muslim University, Aligarh, India, for providing necessary laboratory facilities and also to Prof. John E. Halver for supporting the Fish Nutrition Research Programme at this laboratory. We also gratefully acknowledge the financial assistance of Maulana Azad National Fellowship-University Grant Commissions for minority students for the year 2010–2011 awarded to one of us (Seemab Zehra) and generous funding received under DST-FIST Programme, New Delhi, in the priority area of Fish Nutrition.

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